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(54) **Rhamnogalacturonan acetylesterase (RGAE) from Aspergillus aculeatus**

Rhamnogalacturonan acetylesterase (RGAE) von Aspergillus aculeatus

Rhamnogalacturonan acetylesterase (RGAE) d'Aspergillus aculeatus

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- **Carbohydrate Research, Volume 206, 1990, HENK A. SCHOLS et al., "Rhamnogalacturonase: a Novel Enzyme that Degrades the Hairy Regions of Pectins", pages 105-115**

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Description

[0001] The invention comprises a novel enzyme, which is a rhamnogalacturonan acetyl Esterase (the abbreviation RGAE will usually be used in the following), a corresponding DNA sequence, a vector, a transformed host, a method for production of an RGAE, an enzyme preparation, and a use of the RGAE.

[0002] The invention provides the characterization, the detection and description of a novel RGAE, a partial amino acid sequence of this enzyme, partial DNA sequences, and a total amino acid sequence and a total DNA sequence.

[0003] RGAE is a hydrolase with the systematic enzyme name rhamnogalacturonan acetic-ester acetylhydrolase, which belongs to the group of acetyl esterases (EC no. 3.1.1.6), which catalyze the hydrolysis of acetic esters to the corresponding alcohols and acetate.

BACKGROUND OF THE INVENTION

[0004] Polysaccharides (e.g. pectins) from plants are frequently substituted with acetyl groups (Rombouts, F.M., J. F. Thibault, C. Mercier, "Oxidative enzyme-catalyzed crosslinking of beet pectins", US Patent No. 4,672,034). In the applications of polysaccharides these substitutions influence the gelation properties (Williamson G., C.B. Faulds; JA Matthew, D.B. Archer, V.J. Morris, G.J. Brownsey, M.J, Ridout, "Gelation of sugarbeet and citrus pectins using enzymes extracted from orange peel", Carbohydrate Polymers 13, 387-397, 1990). In the processing of plant material, e.g. fruits and vegetables, endogenous enzymes are used as processing aids to improve yield and quality of the end product (Pilnik, W., A.G.J. Voragen., "Effect of enzyme treatment on the quality of processed fruits and vegetables", in: Jen J. J., "Quality factors of fruits and vegetables, chemistry and technology", ACS Symp. Ser. 405. American Chemical Society, Washington DC, 250-269, 1989). Schols et al. isolated and characterized from apple cell walls an acidic polymeric pectin fragment by the use of a technical enzyme preparation containing pectolytic, hemicellulolytic and cellulolytic enzymes. This enzyme resistant polysaccharide, called "modified hairy region" (MHR) consists of a highly branched rhamnogalacturonan backbone, with acetyl groups on the galacturonic acid residues (Schols, H.A., M.A. Posthumus, A.G.J. Voragen, "Structural features of hairy regions of pectins isolated from apple juice produced by the liquefaction process", Carbohydrate Research, 206, 117-129, 1990). Extensive screening of commercial enzyme preparations have led to an *Aspergillus aculeatus* preparation, which was able to degrade MHR. A novel enzyme called rhamnogalacturonase (RG) was identified and purified from this preparation. During the purification of RG it became obvious that the enzyme works only on saponified MHR and that therefore esterases, particularly acetyl esterases, must play an important role forthe degradation of MHR (Schols, H.A., C.C.J.M. Geraeds, M.J.F. Searle-van Leuwen, F.J.M. Kormelink, A.G.J. Voragen, "Rhamnogalacturonase: a novel enzyme that degrades the hairy regions of pectins", Carbohydrate research 206, 105-115, 1990). Enzymes which can deacetylate branched rhamnogalacturonans, like HMR, are therefore needed, as the high degree of acetylation on branched rhamnogalacturonans hinders the action of enzymes with higher activity on deacetylated rhamnogalacturonans.

[0005] Several polysaccharides (xylan, mannan and pectin) are known to be acetylated, and the acetyl esterases are known to be very specific against their specific polysaccharide substrate, but some of them exhibit activity on non-polysaccharide substrates, like triacetin and naphthol acetate. An *Aspergillus niger* acetylesterase, active towards triacetin and beet pectin, has been described by Mathew et al. (Mathew, J.A., S.J. Howson, M.H.J. Keenan, P.S. Belton, "Improvement of the gelation properties of sugarbeet pectin following treatment with an enzyme preparation derived from *Aspergillus niger* - Comparison with a chemical modification", Carbohydrate Polymers 12, 295-306, 1990). Pectin acetylesterase, highly active towards triacetin, has been purified from citrus peel (Wiliamson, G., "Purification and characterisation of pectin acetyl esterase from orange peel", Phytochemistry 30, 445-449, 1991). Activity on HMR has not been demonstrated for any of these prior art polysaccharide acetyl esterases.

[0006] Thus, the ability of acetyl esterases to hydrolyze the acetyl groups of HMR have not been demonstrated for any of the prior art acetyl esterases, and it is the purpose of the invention to provide an RGAE with high specificity towards HMR.

[0007] The RGAE according to the invention is characterized by the fact that it is immunologically reactive with an antibody raised against a purified RGAE derived from *Aspergillus aculeatus*, CBS 101.43.

[0008] In the present context, the term "derived from" is intended not only to indicate an RGAE produced by strain CBS 101.43, but also an RGAE encoded by a DNA sequence isolated from strain CBS 101.43 and produced in a host organism transformed with said DNA sequence.

[0009] A preferred embodiment of the RGAE according to the invention exhibits the following partial amino acid sequence

```
1                5                  10                  15
Asp-Arg-Val-Tyr-Leu-Ala-Gly-Asp-Ser-Thr-Met-Thr-Lys-Asn-Gly-

                 20                  25
Gly-Xaa-Ser-Gly-Thr-Asn-Gly-Trp-Gly-Glu-Tyr-Leu-Ala-
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(SEQ ID No. 1)

or a partial amino acid sequence homologous thereto, this partial amino acid sequence being part of a polypeptide with RGAE activity.

[0010] In the present context, the term "homologue" is intended to indicate a polypeptide encoded by DNA which hybridizes to the same probe as the DNA coding for the RGAE enzyme under certain specified conditions (such as presoaking in 5xSSC and prehybridizing for 1 hour at ~40°C in a solution of 5xSSC, 5xDenhardt's solution, 50 mM sodium phosphate, pH 6.8, and 50 µg of denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 50 µCi 32-P-dCTP labelled probe for 18 hours at ~40°C followed by washing three times in 2×SSC, 0.2% SDS at 40°C for 30 minutes). More specifically, the term is intended to refer to a DNA sequence which is at least 70% homologous to the sequence shown above encoding the RGAE of the invention. The term is intended to include modifications of the DNA sequence indicated above, such as nucleotide substitutions which do not give rise to another amino acid sequence of the RGAE but which correspond to the codon usage of the host organism into which the DNA construct is introduced or nucleotide substitutions which do give rise to a different amino acid sequence and therefore, possibly, a different protein structure which might give rise to an RGAE mutant with different properties than the native enzyme. Other examples of possible modifications are insertion of one or more nucleotides into the sequence, addition of one or more nucleotides at either end of the sequence, or deletion of one or more nucleotides at either end or within the sequence.

[0011] Thus, surprisingly it has been found that the RGAE according to the invention is highly specific for the deacetylation of MHR, but that it does not show any acitivity towards triacetin and beet pectin, and also it exhibits a higher specificity than the prior art acetyl esterases.

[0012] The above indicated partial amino acid sequence can be used for construction of DNA probes which can be used for screening a genomic library for organisms expressing such enzyme, or a cDNA library, thereby obtaining DNA sequences, which can be used either for an overproduction of RGAE, if inserted in the microorganism species, from which the parent DNA molecule originated,. or for production of RGAE without accompanying closely related enzymes, if inserted in a host microorganism, which in its not-transformed condition does not produce any enzymes closely related to RGAE. The DNA sequences can be established otherwise, as will apppear from the following.

[0013] Thus, the purpose of the invention is the provision of a new RGAE and of means and methods for production of RGAE in better yield and higher purity than hitherto possible, and of a use of RGAE either alone or in combination with other significant amounts of enzymes for degradation of plant cell wall tissue, more efficient than hitherto possible. Also it is the purpose of the invention to provide novel products, wherein the proportion of the RGAE is either increased or decreased in relation to the proportion in the original product.

[0014] The recombinant DNA sequence obtainable according to the invention comprises a DNA sequence coding for a polypeptide having RGAE activity, or a DNA sequence having substantial sequence homology to such RGAE coding sequence.

[0015] In the following it will be explained in detail how the recombinant DNA sequence according to the invention can be produced.

[0016] Crude enzyme preparations produced from *Aspergillus aculeatus* for purification of the RGAE can be produced as follows. For the sake of brevity this crude *Aspergillus aculeatus* preparation will be referred to in the following as A. a.e.p.

[0017] The strain *Aspergillus aculeatus* CBS 101.43 as a gene donor was fermented in a pilot plant scale in the following way.

[0018] An agar substrate with the following composition was prepared in a Fembach flask:

Peptone Difco	6 g
Aminolin Ortana	4 g
Glucose	1 g
Yeast extract Difco	3 g
Meat extract Difco	1.5 g

(continued)

KH_2PO_4 Merck	20 g
Malt extract Evers	20 g
Ion exchanged H_2O	ad 1000 ml

[0019] pH was adjusted to between 5.30 and 5.35. Then 40 g of Agar Difco was added, and the mixture was autoclaved for 20 minutes at 120°C (the substrate is named E-agar).

[0020] The strain CBS 101.43 was cultivated on an E-agar slant (37°C). The spores from the slant were suspended in sterilized skim-milk, and the suspension was lyophilized in vials. The contents of one lyophilized vial was transferred to the Fembach flask. The flask was then incubated for 13 days at 30°C.

[0021] A substrate with the following composition was prepared in a 500 litre seed fermenter:

$CaCO_3$	1.2 kg
Glucose	7.2 kg
Rofec (corn steep liquor dry matter)	3.6 kg
Soy bean oil	1.2 kg

[0022] Tap water was added to a total volume of around 240 litres. pH was adjusted to around 5.5 before addition of $CaCO_3$. The substrate was sterilized in the seed fermenter for 1 hour at 121°C. Final volume before inoculation was around 300 litres.

[0023] The Fernbach flask spore suspension was transferred to the seed fermenter. Seed fermentation conditions were:

[0024] Fermenter type: Conventional aerated and agitated fermenter with a height/diameter ratio of around 2.3.

Agitation	300 rpm (two turbine impellers)
Aeration	300 normal litre air per minute
Temperature	30 to 31°C
Time	around 28 hours

[0025] Around 28 hours after inoculation 150 litres was transferred from the seed fermenter to the main fermenter.

[0026] A substrate with the following composition was prepared in a 2500 litre main fermenter:

Toasted soy meal	90 kg
KH_2PO_4	20 kg
Pluronic® antifoam agent	150 ml

[0027] Tap water was added to a total volume of around 900 litres. The toasted soy meal was suspended in water. pH was adjusted to 8.0 with NaOH, and the temperature was raised to 50°C. Thereafter around 925 Anson units of Alcalase® 0.6 L was added to the suspension. The mixture was held for 4 hours at 50°C and pH = 8.0 (Na_2CO_3 addition) with no aeration and 100 rpm agitation. Thereafter the remaining substrate components were added and pH was adjusted to around 6.0 with phosphoric acid. The substrate was sterilized in the main fermenter for 1½ hours at 123°C. Final volume before inoculation was around 1080 litres.

[0028] Then 150 litres of seed culture was added.

[0029] Fermentation conditions were:

[0030] Fermenter type: Conventional aerated and agitated fermenter with a height/diameter ratio of around 2.7.

Agitation	250 rpm (two turbine impellers)
Aeration	1200 normal litre air per minute
Temperature	30°C
Time	around 151 hours

[0031] From 24 fermentation hours to around 116 fermentation hours pectin solution was added aseptically to the main fermenter at a constant rate of around 8 litres per hour. The pectin solution with the following composition was prepared in a 500 litre dosing tank:

Pectin genu*)	22 kg
Phosphoric acid, conc.	6 kg
Piuronic® antifoam agent	50 ml

*) Genu pectin (citrus type NF from the Copenhagen pectin factory Ltd.)

[0032] Tap water was added to a total volume of around 325 litres. The substrate was sterilized in the dosing tank for 1 hour at 121°C. Final volume before start of dosage was around 360 litres. When this portion ran out, another similar portion was made. Total volume of pectin solution for one fermentation was around 725 litres.

[0033] After around 151 fermentation hours the fermentation process was stopped. The around 1850 litres of culture broth were cooled to around 5°C and the enzymes were recovered according to the following method.

[0034] The culture broth was drum filtered on a vacuum drum filter (Dorr Oliver), which was precoated with Hyflo Super-Cell diatomaceous earth (filter aid). The filtrate was concentrated by evaporation to around 15% of the volume of the culture broth. The concentrate was filtered on a Seitz filter sheet (type supra 100) with 0.25% Hyflo Super-Cell as a filter aid (in the following table referred to as filtration I). The filtrate was precipitated with 561 g of $(NH_4)_2SO_4$/l at a pH of 5.5, and 4% Hyflo Super-Cell diatomaceous earth is added as a filter aid. The precipitate and the filter aid are separated by filtration on a frame filter. The filter cake is dissolved in water, and insoluble parts are separated by filtration on a frame filter. The filtrate is check filtered on a Seitz filter sheet (type supra 100) with 0.25% Hyflo Super-Cell as a filter aid (in the following table referred to as filtration II). The filtrate is diafiltered on an ultrafiltration apparatus. After diafiltration the liquid is concentrated to a dry matter content of 12.7% (in the following table referred to as dry matter content in concentrate).

[0035] A facultative base treatment for partial removal of the protease activity can be carried out at this stage. In case the base treatment is used it is carried out at a pH of 9.2 for 1 hours, whereafter the pH value is adjusted to 5.0.

[0036] Now the liquid is check filtered and filtered for the purpose of germ reduction and the filtrate is freeze-dried on a freeze-drying equipment from Stokes.

[0037] The pure RGAE is obtainable from the A.a.e.p. as shown in Table 1.

Table 1 RHAMNOGALACTURONAN-ACETYL ESTERASE PURIFICATION

Aspergillus aculeatus enzyme broth

1: ULTRAFILTRATION - DIALYSIS
Filtron Minisette, filter area 3500 cm^2, membrane NMWL 10,000
20mM TRIS, pH 5.0; 5 x volume

↓

2: IEC: WATER ACCELL QMA-PLUS, Fig. 1
(column: 5.0 x 23.0 cm, flow 60 ml/minute)
eluent = 20mM TRIS, pH 5.0, increasing NaCl-gradient:
0.0M-linear-0.0125M-linear-0.25M-linear-0.5M

↓

3: ULTRAFILTRATION - DIALYSIS
Filtron Minisette, filter area 3500 cm^2, membrane NMWL 10,000
20mM TRIS, pH 4.2; 5 x volume

↓

4: CROSSLINKED ALGINATE, Fig. 2
(column: 4.9 x 17.5 cm, flow 10 ml/minute)
eluent 1 = 20mM TRIS, pH 4.2; eluent 2 = 20 mM TRIS, pH 6.0

↓

5: SAMPLE PREPARATION
crosslinked alginate pool, addition of solid $(NH_4)_2SO_4$ to
2 M concentration and pH adjustment to 5.0

↓

6: HIC: PHENYL TOYOPEARL 650 (M), Fig. 3
(column: 5.0 x 25.0 cm, flow 60 ml/minute)
eluent: water, decreasing $(NH_4)_2SO_4$-gradient:
2M-concave decrease (=linear decrease of conductivity)-0.5M-step-0.0M

↓

7: ULTRAFILTRATION - DIALYSIS
Filtron Minisette, filter area 3500 cm^2, membrane NMWL 10,000
20 mM TRIS, pH 5.0; 5 x volume

↓

8: IEC: PROTEIN PAC DEAE-8HR, Fig. 4
(column. 2.0 x 10.0 cm, flow 4.5 ml/minute)
eluent: 20 mM TRIS, pH 5.0; increasing NaCl-gradient:
0.0M-step-0.05M-linear-0.1M-linear-0.15M

↓

9: SAMPLE PREPARATION
Protein Pac DEAE-8HR pool, dilution with 20mM TRIS to 2 x volume
(decrease of NaCl-molarity for next step)

↓

10: IEC: PROTEIN PAC DEAE-8HR, Fig. 5
(column: 2.0 x 10.0 cm, flow 4.5 ml/minute)
eluent: 20mM TRIS, pH 5.0; increasing NaCl-gradient:
20mM-step-60mM-linear-100mM-linear-150mM

↓

RHAMNOGALACTURONAN-ACETYL ESTERASE

ad 1:
Buffer exchange in order to prepare for step 2, removal of small particles and about 50% of the colour, dilution to max. 15 mg protein/ml (otherwise the sample will not bind to the column in step 2).

ad 2:
IEC is ion exchange chromatography. The acetylesterease fraction was pooled from 0.04 - 0.08 M NaCl.

ad 3:
Concentration and buffer exchange in order to prepare for step 4.

ad 4:
Affinity chromatography - the non retained fraction was pooled. The preparation of the crosslinked alginate was done according to Rombouts F.M., C.C.J.M. Geraeds, J. Visser, W. Pilnik, "Purification of various pectic enzymes on crosslinked polyuronides", in: Gribnau, T.C.J., J. Visser, R.J.F. Nivard (Editors), Affinity Chromatography and Related Techniques, Elsevier Scientific Publishing Company, Amsterdam, 255-260, 1982.

ad 5:
Buffer adaption in order to prepare for step 6.

ad 6:
HIC is hydrophobic interaction chromatography. The acetyl esterase fraction was pooled from 1.0M - 0.9M $(NH_4)_2SO_4$.

ad 7:
Concentration and buffer exchange in order to prepare for step 8.

ad 8:
IEC is ion exchange chromatography. The acetyl esterase fraction was pooled from 65mM - 70mM NaCl.

ad 9:
Buffer adaption in order to prepare for step 10.

ad 10:
IEC is ion exchange chromatography. The acetyl esterase fraction was pooled from 65mM- 70mM NaCl.

[0038] The thus purified RGAE may be employed for immunization of animals for the production of antibodies. More specifically, antiserum against the RGAE of the invention may be raised by immunizing rabbits (or other rodents) according to the procedure described by N. Axelsen et al. in: A Manual of Quantitative Immunoelectrophoresis, Blackwell Scientific Publications, 1973, Chapter 23, or A. Johnstone and R. Thorpe, Immunochemistry in Practice, Blackwell Scientific Publications, 1982 (more specifically pp. 27-31). Purified immunoglobulins may be obtained from the antisera, for example by salt precipitation ($(NH_4)_2 SO_4$), followed by dialysis and ion exchange chromatography, e.g. on DE-AE-Sephadex. Immunochemical characterization of proteins may be done either by Ouchterlony double-diffusion analysis (O. Ouchterlony in: Handbook of Experimental Immunology (D.M. Weir, Ed.), Blackwell Scientific Publications, 1967, pp. 655-706), by crossed immunoelectrophoresis (N. Axelsen et al., supra, Chapters 3 and 4), or by rocket immunoelectrophoresis (N. Axelsen et al., Chapter 2,).

Amino acid sequence

[0039] The previously indicated partial amino acid sequence was determined from the purified RGAE by means of automated sequencing (Applied Biosystems 473A protein sequencer). On the basis of the sequence the purity of the sample is estimated to be more than 90%.

[0040] The RGAE is further characterized, as indicated in the following.

[0041] The RGAE has its optimal activity at pH 5.5 and at a temperature of 40°C. At 50°C and pH 5.0 no activity loss could be observed within 20 hours. At 30°C the pH-stability was highest at pH 6-7.

[0042] The RGAE has a specific activity towards MHR and released a maximum of about 70% of all acetyl groups present in MHR.

[0043] Combinations of this RGAE with pure pectin methylesterase of both citrus and fungal origin, pure exo- and endo-arabinases from *Aspergillus* species (*Aspergillus niger, Aspergillus aculeatus*) did not provide any increase in

acetyl release from either beet pectin or apple MHR.

[0044] Pretreatment of these substrates, in order to remove arabinose side chains, did not exhibit any stimulating effect either. From these results it appears that a novel RGAE has been identified, which is highly specific for the acetyl esters of ramified pectic regions.

Molecular weight: approx. 31,000 to 35,000 Daltons
Isoelectric point: pH 4.2

[0045] A preferred embodiment of the RGAE according to the invention is characterized by the fact that the RGAE exhibits a pH-optimum of 4.0 - 7.0, preferably 4.5 - 6.0, an isoelectric point of 3.7 - 6.7, preferably 4.0-4.5, a molecular weight of between 30,000 and 50,000 Dalton, and a temperature optimum between 10 and 50°C, preferably between 25 and 45°C.

[0046] Also the invention comprises a recombinant DNA sequence, which is characterized by encoding for the RGAE according to the invention.

[0047] A preferred embodiment of the recombinant DNA sequence according to the invention is characterized by the fact that it comprises a DNA sequence selected from

a) the *Aspergillus aculeatus* RGAE DNA insert
b) a DNA sequence which hybridizes to the coding region for the mature RGAE DNA comprised by the DNA insert of a) and which comprises a structural gene for a polypeptide with RGAE activity, and optionally a promoter, a coding region for a signal or leader peptide and/or transcriptional terminator.
c) a derivative of a DNA sequence defined in a) or b), or
d) a DNA sequence which codes for a mature RGAE or a signal peptide or a leader peptide thereof and which is degenerate within the meaning of the genetic code with respect to a DNA sequence of a) or b).

[0048] A preferred embodiment of the recombinant DNA sequence according to the invention is characterized by the fact that it comprises the following partial DNA sequences

a) ATGAAGACCG CCGCCTCTTG CACCGCTCTT CTTCCTCCCC TCTGCCCTCG CCACGACNNG (SEQ ID No. 2)

b) GTCTATCTCG CGGGTGACTC GACCATGGCC AAGAATGGAG GCGGGTCGGG AACTAACGGC (SEQ ID No. 3)

c) TGGGGCGAGT ACCTGCGAGT TACCTCTCCG CGACAGTGGT TAACGACGCG GTCGCG (SEQ ID No. 4)

d) GACGCAACCT ATGAAGACCT TGGAATGCCA CCGTCAACTC GTATTCCCCA TCGATCACAC (SEQ ID No. 5)

e) CCACACCAGT CCTGCGGCGC GAGGTCGTGG CTGAGCGTTC TTGAAGGCGG TGGTATGCAC (SEQ ID No. 6)

f) GGGTACGTCG TTGAAGAGTG TGTTGACGAC GACGAGCTT (SEQ ID No. 7)

[0049] A preferred embodiment of the recombinant DNA sequence according to the invention is characterized by the fact that it comprises the following DNA sequences

a) ATGAAGACCG CCGCCTCTTG CACCGCTCTT CTTCCTCCCC TCTGCCCTCG
CCACGACNNG GTCTATCTCG CGGGTGACTC GACCATGGCC AAGAATGGAG
GCGGGTCGGG AACTAACGGC TGGGGCGAGT ACCTGCGAGT TACCTCTCCG
CGACAGTGGT TAACGACGCG GTCGCG (SEQ ID No. 8)

b) GACGCAACCT ATGAAGACCT TGGAATGCCA CCGTCAACTC GTATTCCCCA
TCGATCACAC CCACACCAGT CCTGCGGCGC GAGGTCGTGG CTGAGCGTTC
TTGAAGGCGG TGGTATGCAC GGGTACGTCG TTGAAGAGTG TGTTGACGAC
GACGAGCTT (SEQ ID No. 9)

[0050] A preferred embodiment of the recombinant DNA sequence according to the invention is characterized by the fact that it comprises

AGTCTATAAG AAGATTGACA GCCAAGAACA CCACCCACAA TGAAGACCGC
CGCCTCTTGC ACCGCTCTTC TTCCTCCCCT CTGCCCTCGC CACGACNNGG
TCTATCTCGC GGGTGACTCG ACCATGGCCA AGAATGGAGG CGGGTCGGGA
ACTAACGGCT GGGGCGAGTA CCTCGCCAGT TACCTCTCCG CGACAGTGGT
TAACGACGCG GTCGCGGGCC GCAGCGCGCG CTCGTACACA CGCGAGGGTC
GGTTCGAGAA CATCGCCGAT GTAGTGACGG CGGGCGACTA CGTGATCGTC
GAGTTCGGCC ACAACGACGG TGGCTCGCTG TCCACGGACA ATGGACGCAC
CGACTGCTCC GGTACCGGGG CAGAAGTCTG CTATAGCGTC TACGACGGGG
TCAACGAGAC CATCCTCACC TTCCCCGCCT ACCTGGAGAA CGCCGCCAAG
CTGTTCACCG CCAAGGGCGC CAAGGTCATT CTCAGCAGCC AGACCCCCAA
CAACCCCTGG GAGACCGGCA CCTTCGTCAA CTCCCCCACG CGCTTCGTTG
AGTACGCCGA GCTGGCCGCC GAGGTCGCTG GCGTCGAGTA CGTCGACCAC
TGGTCCTACG TGGACAGCAT CTATGAGACC TTGGCAATGC CACCGTCAAC
TCGTATTCCC CATCGATCAC ACCCACACCA GTCCTGCGGC GCGAGGTCGT
GGCTGAGCGT TCTTGAAGGC GGTGGTATGC ACGGGTACGT CGTTGAAGAG
TGTGTTGACG ACGACGAGCT TTGAGGGGAC ATGTCTGTGA TTGAGCAGAT
GGAAAGACAA AGGAGTGGAC GGATAAGGAC AGGAGTTGTC ATGTATAGTG
GTAGTTTGTG CATTGCAAAT GGTATCTGAA CTGGCTCGCT TATGCTCATG
ATCGACAAAA AAAAAAAAAA AAAAAAAAAA AAAAA (SEQ ID NO. 10)

or a sequence homologous thereto encoding for an RGAE according to the invention.

[0051] Also, the invention comprises a vector which is characterized by the fact that it comprises the recombinant DNA sequence according to the invention.

[0052] A preferred embodiment of the vector according to the invention is characterized by the fact that the promoter is the *Aspergillus oryzae* takaamylase promoter.

[0053] Also the invention comprises a transformed host which is characterized by the fact that it contains the vector according to the invention.

[0054] A preferred embodiment of the transformed host according to the invention is characterized by the fact that the transformed host is an *Aspergillus* strain. Hereby a good production capacity of the RGAE is obtained.

[0055] A preferred embodiment of the transformed host according to the invention is characterized by the fact that the transformed host is a strain belonging to the species *Aspergillus aculeatus, Aspergillus niger, Aspergillus oryzae* or *Aspergillus awamori*. Hereby a good production capacity of the RGAE is obtained.

[0056] A preferred embodiment of the transformed host according to the invention is characterized by the fact that the transformed host is a microorganism, which in its non-transformed condition does not produce RGAE or only produces RGAE in insignificant amounts, preferably *Bacillus sp., E. coli* or *S. cerevisiae*. Hereby a "tailor made" enzyme preparation with high RGAE activity and a spectrum of other wanted specific enzyme activities can be obtained.

[0057] Also, the invention comprises a method for production of an RGAE by use of a transformed host according to the invention. By means of this method the RGAE can be obtained in high yield.

[0058] Also, the invention comprises the RGAE, when produced by the method according to the invention. RGAE can be obtained in high yield.

[0059] Also, the invention comprises an enzyme preparation which is characterized by the fact that it contains a pectinase preparation usable for degradation or modification of plant cell walls enriched with the RGAE according to the invention. In this manner a boosting of the cell wall degrading ability of the pectinase preparation can be obtained.

[0060] A preferred embodiment of the enzyme preparation according to the invention is characterized by the fact that the pectinase preparation is producible by means of a microorganism belonging to the genus *Aspergillus*, preferably *Aspergillus niger, Aspergillus aculeatus, Aspergillus awamori* or *Aspergillus oryzae*. Such preparation is able to provide an extraordinary good decomposition of vegetable cell walls.

[0061] A preferred embodiment of the enzyme preparation according to the invention is characterized by the fact that the RGAE is the RGAE produced by means of the method according to the invention. The production costs of this preparation are relatively low.

[0062] Also, the invention comprises a use of the RGAE according to the invention as an agent for degradation or modification of acetylated rhamnogalacturonan.

[0063] A preferred embodiment of the use of the RGAE according to the invention is a use as an agent for degradation or modification of plant cell walls. At present, degradation of plant cell walls is the most preferred use of the RGAE according to the invention, due to the high plant cell wall degradation activity.

[0064] A preferred embodiment of the use of the RGAE according to the invention is a use where the RGAE is used together with enzymes specific to deacylated or partially deacylated MHR. These further enzymes include all enzymes, which attack deacetylated and partially deacetylated ramified Rhamnogalacturonans with a higher specificity than they attack acetylated rhamnogalacturonans, including enzymes which attack the Rhamnogalacturonan backbone by endo or exo attack, or enzymes which attack the side branches.

[0065] Also the invention comprises a use of the enzyme preparation according to the invention as an agent for degradation or modification of acetylated rhamnogalacturonanes.

[0066] A preferred embodiment of the use of the enzyme preparation according to the invention is a use as an agent for degradation or modification of plant cell walls. At present, degradation of plant cell walls is the most preferred use of the enzyme preparation according to the invention, due to the high plant cell wall degradation activity.

[0067] A preferred embodiment of the use of the enzyme preparation according to the invention is a use where the enzyme preparation is used together with enzymes specific to deacylated or partially deacylated MHR. These further enzymes include all enzymes, which attack deacetylated and partially deacetylated ramified Rhamnogalacturonans with a higher specificity than they attack acetylated Rhamnogalacturonans, including enzymes which attack the Rhamnogalacturonan backbone by endo or exo attack, or enzymes which attack the side branches.

[0068] Fig. 6 is a map of plasmid pYHD17, wherein "TPI promoter" indicates the *S. cerevisiae* triose phosphate isomerase promoter, "Terminator" indicates the transcription terminator, "Amp" indicates the gene mediating ampicillin resistance, "2μ ori" indicates the yeast plasmid 2 μ origin of replication, and "URA3" indicates a gene encoding a selection marker complementing a uracil deficiency in the host strain.

EXAMPLES

Materials and methods

[0069] Donor organism: mRNA was isolated from *Aspergillus aculeatus*, CBS 101.43, grown in a soy-containing fermentation medium with agitation to ensure sufficient aeration. Mycelia were harvested after 3-5 days' growth, immediately frozen in liquid nitrogen and stored at - 80°C.

[0070] Yeast strains: The *Saccharomyces cerevisiae* strain used was yNG231 (MAT alpha, leu2, ura3-52, his4-539, pep4-delta 1, cir+) or JG169 (MATα; ura 3-52; leu 2-3, 112; his 3-D200; pep 4-113; prc1::HIS3; prb1:: LEU2; cir+).

Construction of an expression plasmid

[0071] The commercially available plasmid pYES II (Invitrogen) was cut with SpeI, filled in with Klenow DNA polymerase + dNTP and cut with ClaI. The DNA was size fractionated on an agarose gel, and a fragment of about 2000 bp was purified by electroelution. The same plasmid was cut with ClaI/PvuII, and a fragment of about 3400 bp was purified by electroelution. The two fragments were ligated to a blunt-ended SphI/EcoRI fragment containing the yeast TPI promoter. This fragment was isolated from a plasmid in which the TPI promoter from *S. cerevisiae* (cf. T. Albers and G. Kawasaki, *J. Mol. Appl. Genet. 1*, 1982, pp. 419-434) was slightly modified: an internal SphI site was removed by deleting the four bp constituting the core of this site. Furthermore, redundant sequences upstream of the promoter were removed by Bal1 exonuclease treatment followed by addition of a SphI linker. Finally, an EcoRI linker was added at position -10. After these modifications, the promoter is included in a SphI-EcoRI fragment. Its Efficiency compared to the original promoter appears to be unaffected by the modifications. The resulting plasmid pYHD17 is shown in Fig. 6.

Preparation of RNase-free glassware, tips and solutions

[0072] All glassware used in RNA isolations was baked at + 220°C for at least 12 h. Eppendorf tubes, pipet tips and plastic columns were treated in 0.1% diethylpyrocarbonate (DEPC) in EtOH for 12 hours, and autoclaved. All buffers and water (except Tris-containing buffers) were treated with 0.1% DEPC for 12 hours at 37°C, and autoclaved.

Extraction of total RNA

[0073] The total RNA was prepared by extraction with guanidinium thiocyanate followed by ultracentrifugation through a 5.7 M CsCl cushion (Chirgwin *et* al., 1979) using the following modifications. The frozen mycelia were ground in liquid N_2 to fine powder with a mortar and a pestle, followed by grinding in a precooled coffee mill, and immediately suspended in 5 vols of RNA extraction buffer (4 M GuSCN, 0.5% Na-laurylsarcosine, 25 mM Na-citrate, pH 7.0, 0.1 Mβ-mercaptoethanol). The mixture was stirred for 30 minutes at RT and centrifuged (30 minutes, 5000 rpm, RT, Heraeus Megafuge 1.0 R) to pellet the cell debris. The supernatant was collected, carefully layered onto a 5.7 M CsCl cushion (5.7 M CsCl, 0.1 M EDTA, pH 7.5, 0.1% DEPC; autoclaved prior to use) using 26.5 ml supernatant per 12.0 ml CsCl cushion, and centrifuged to obtain the total RNA (Beckman, SW 28 rotor, 25,000 rpm, RT, 24h). After centrifugation the supernatant was carefully removed and the bottom of the tube containing the RNA pellet was cut off and rinsed with 70% EtOH. The total RNA pellet was transferred into an Eppendorf tube, suspended in 500 µl TE, pH 7.6 (if difficult, heat occasionally for 5 minutes at 65°C), phenol extracted and precipitated with ethanol for 12 hours at - 20°C (2.5 vols EtOH, 0.1 vol 3M NaAc, pH 5.2). The RNA was collected by centrifugation. washed in 70% EtOH, and resuspended in a minimum volume of DEPC-DIW. The RNA concentration was determined by measuring $OD_{260/280}$.

Isolation of poly(A)$^+$RNA

[0074] The poly(A)$^+$ RNAs were isolated by oligo(dT)-cellulose affinity chromatography (Aviv & Leder, 1972). Typically, 0.2 g of oligo(dT) cellulose (Boehringer Mannheim) was preswollen in 10 ml of 1 x column loading buffer (20 mM Tris-Cl, pH 7.6, 0.5 M NaCl, 1 mM EDTA, 0.1% SDS), loaded onto a DEPC-treated, plugged plastic column (Poly Prep Chromatography Column, Bio Rad), and equilibrated with 20 ml 1 x loading buffer. The total RNA was heated at 65°C for 8 minutes, quenched on ice for 5 minutes, and after addition of 1 vol 2 x column loading buffer to the RNA sample loaded onto the column. The eluate was collected and reloaded 2-3 times by heating the sample as above and quenching on ice prior to each loading. The oligo(dT) column was washed with 10 vols of 1 x loading buffer, then with 3 vols of medium salt buffer (20 mM Tris-Cl, pH 7.6, 0.1 M NaCl, 1 mM EDTA, 0.1% SDS), followed by elution of the poly(A)$^+$ RNA with 3 vols of elution buffer (10 mM Tris-Cl, pH 7.6, 1 mM EDTA, 0.05% SDS) preheated to + 65°C, by collecting 500 µl fractions. The OD_{260} was read for each collected fraction, and the mRNA containing fractions were pooled and ethanol precipitated at - 20°C for 12 h. The poly(A)$^+$ RNA was collected by centrifugation, resuspended in DEPC-DIW and stored in 5-10 µg aliquots at - 80°C.

Northern blot analysis

[0075] The poly(A)$^+$ RNAs (5 µg/sample) from various mycelia were electrophoresed in 1.2 agarose-2.2 M formaldehyde gels (Sambrook et at., 1989) and blotted to nylon membranes (Hybond-N, Amersham) with 10 x SSC (Sambrook et al., 1989) as transfer buffer. Three random-primed (Feinberg & Vogelstein, 1983) ^{32}P-labeled cDNA probes were used in individual hybridizations: 1) a 1.3 kb Not I-Spe I fragment for polygalacturonase I from *A. aculeatus*, 2) a 1.3 kb Not I-Spe I fragment encoding endoglucanase I from *A. aculeatus* and 3) a 1.2 kb Eag I fragment for galactanase I from *A. aculeatus*. Northern hybridizations were carried out in 5 x SSC (Sambrook et al., 1989), 5 x Denhardt's solution

(Sambrook et al., 1989), 0.5% SDS (w/v) and 100/µg/ml denatured salmon sperm DNA with a probe concentration of ca. 2 ng/ml for 16 hours at 65°C followed by washes in 5 x SSC at 65°C (2x15 minutes), 2 x SSC, 0.5% SDS (1 x 30 minutes), 0.2 x SSC, 0.5% SDS (1 X 30 minutes), and 5 x SSC (2 x 15 minutes). After autoradiography at - 80°C for 12 hours, the probe # 1 was removed from the filter according to the manufacturer's instructions and rehybridized with probe #2, and eventually with probe #3. The RNA ladder from Bethesda Research Laboratories was used as a size marker.

cDNA synthesis:

First strand synthesis

[0076] Double-stranded cDNA was synthesized from 5µg of *A. aculeatus* poly(A)$^+$ RNA by the RNase H method (Gubler & Hoffman 1983, Sambrook et al., 1989) using the hair-pin modification. The poly(A)$^+$RNA (5µg in 5 µl of DEPC-treated water) was heated at 70°C for 8 minutes, quenched on ice, and combined in a final volume of 50 µl with reverse transcriptase buffer (50 mM Tris-Cl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ 10 mM DTT, Bethesda Research Laboratories) containing 1 mM each dNTP (Pharmacia), 40 units of human placental ribonuclease inhibitor (RNasin, Promega), 10µg of oligo$(dT)_{12-18}$ primer (Pharmacia) and 1000 units of Superscript II RNase H-reverse transcriptase (Bethesda Research Laboratories). First-strand cDNA was synthesized by incubating the reaction mixture at 45°C for 1 hour.

Second strand synthesis

[0077] After synthesis 30 µl of 10 mM Tris-Cl, pH 7.5, 1 mM EDTA was added, and the mRNA:cDNA hybrids were ethanol precipitated for 12 hours at - 20°C by addition of 40 µg glycogen carrier (Boehringer Mannheim) 0.2 vols 10 M NH_4Ac and 2.5 vols 96% EtOH. The hybrids were recovered by centrifugation, washed in 70% EtOH, air dried and resuspended in 250 µl of second strand buffer (20 mM Tris-Cl, pH 7.4, 90 mM KCl, 4.6 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 16µM βNAD$^+$) containing 100 µM each dNTP, 44 units of *E. coli* DNA polymerase I (Amersham), 6.25 units of RNase H (Bethesda Research Laboratories) and 10.5 units of *E. coli* DNA ligase (New England Biolabs). Second strand cDNA synthesis was performed by incubating the reaction tube at 16°C for 3 hours, and the reaction was stopped by addition of EDTA to 20 mM final concentration followed by phenol extraction.

Mung bean nuclease treatment

[0078] The double-stranded (ds) cDNA was ethanol precipitated at - 20°C for 12 hours by addition of 2 vols of 96% EtOH, 0.1 vol 3 M NaAc, pH 5.2, recovered by centrifugation, washed in 70% EtOH, dried (SpeedVac), and resuspended in 30 µl of Mung bean nuclease buffer (30 mM NaAc, pH 4.6, 30O mM NaCl, 1 mM $ZnSO_4$, 0.35 mM DTT. 2% glycerol) containing 36 units of Mung bean nuclease (Bethesda Research Laboratories). The single-stranded hair-pin DNA was clipped by incubating the reaction at 30°C for 30 minutes, followed by addition of 70 µl 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, phenol extraction, and ethanol precipitation with 2 vols of 96% EtOH and 0.1 vol 3M NaAc, pH 5.2 at - 20°C for 12 hours.

Blunt-ending with T4 DNA polymerase

[0079] The ds cDNA was blunt-ended with T4 DNA polymerase in 50 µl of T4 DNA polymerase buffer (20 mM Tris-acetate, pH 7.9, 10 mM MgAc, 50 mM KAc, 1 mM DTT) containing 0.5 mM each dNTP and 7.5 units of T4 DNA polymerase (Invitrogen) by incubating the reaction mixture at + 37°C for 15 minutes. The reaction was stopped by addition of EDTA to 20 mM final concentration, followed by phenol extraction and ethanol precipitation.

Adaptor ligation and size selection

[0080] After the fill-in reaction the cDNA was ligated to non-palindromic BstX I adaptors (1 µg/µl, Invitrogen) in 30 µl of ligation buffer (50 mM Tris-Cl, pH 7.8, 10 mM, $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 µg/ml bovine serum albumin) containing 600 pmol BstX I adaptors and 5 units of T4 ligase (Invitrogen) by incubating the reaction mix at + 16°C for 12 hours. The reaction was stopped by heating at + 70°C for 5 minutes, and the adapted cDNA was size-fractionated by agarose gel electrophoresis (0.8% HSB-agarose, FMC) to separate unligated adaptors and small cDNAs. The cDNA was size-selected with a cut-off at 0.7 kb, and the cDNA was electroeluted from the agarose gel in 10 mM Tris-Cl, pH 7.5, 1 mM EDTA for 1 hour at 100 volts, phenol extracted and ethanol precipitated at - 20°C for 12 hours as above.

Construction of cDNA libraries:

[0081] The adapted, ds cDNA was recovered by centrifugation, washed in 70% EtOH and resuspended in 25 ml DIW. Prior to large-scale library ligation, four test ligations were carried out in 10 µl of ligation buffer (same as above) each containing 1 µl ds cDNA (reaction tubes #1 - #3), 2 units of T4 ligase (Invitrogen) and 50 ng (tube #1), 100 ng (tube #2) and 200 ng (tubes #3 and #4) BstX I cleaved yeast expression vector either pYES 2.0 vector Invitrogen or yHD13. The ligation reactions were performed by incubation at + 16°C for 12 hours, heated at 70°C for 5 minutes, and 1 µl of each ligation electroporated (200 Ω, 2.5 kV, 25 µF) to 40 µl competent *E. coli* 1061 cells (OD600 = 0.9 in 1 liter LB-broth, washed twice in cold DIW, once in 20 ml of 10% glycerol, resuspended in 2 ml 10% glycerol). After addition of 1 ml SOC to each transformation mix, the cells were grown at + 37°C for 1 hour, 50 µl plated on LB + ampicillin plates (100 µg/ml) and grown at + 37°C for 12 hours.

[0082] Using the optimal conditions a large-scale ligation was set up in 40 µl of ligation buffer containing 9 units of T4 ligase, and the reaction was incubated at + 16°C for 12 hours. The ligation reaction was stopped by heating at 70°C for 5 minutes, ethanol precipitated at - 20°C for 12 hours, recovered by centrifugation and resuspended in 10 µl DIW. One µl aliquots were transformed into electrocompetent *E. coli* 1061 cells using the same electroporation conditions as above, and the transformed cells were titered and the library plated on LB + ampicillin plates with 5000-7000 c.f.u./plate. To each plate was added 3 ml of medium. The bacteria were scraped off, 1 ml glycerol was added and stored at - 80°C as pools. The remaining 2 ml were used for DNA isolation. If the amount of DNA was insufficient to give the required number of yeast transformants, large scale DNA was prepared from 500 ml medium (TB) inoculated with 50 µl of - 80°C bacterial stock propagated overnight.

Generation of a cDNA probe by polymerase chain reaction

[0083] To obtain a cDNA probe for rhamnogalacturonan acetylesterase I from *A. aculeatus* RE4, a degenerate oligonudeotide (RGAE/s_2, Fig. 7) corresponding to a region in the NH_2-terminal sequence of the purified enzyme was synthesized by incorporating deoxyinosines at four of the ambiguous positions. Fig. 7 shows the deduced, deoxyinosine-containing primer sequence used in the PCR aligned with the corresponding amino acid sequence obtained from purified rhamnogalacturonan acetylesterese I from *Aspergillus aculeatus.* The primer was used pairwise with the direct (primer #22, 5'-CTGTAATACGACTCACTA -3') and reverse (primer #43, 5'-ATTACATGATGCGGCCCT -3') pYES 2.0 primers to amplify the target RGAE cDNA from an amplified cDNA library pool containing 7000 clones employing the polymerase chain reaction technique (Ohara *et al.* 1989). The PCR reactions were carried out in 100 µl PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01%, Perkin-Elmer, Cetus) containing 550 pmol of sense primer (RGAE/s_2) and 800 pmol of each antisense primer (see above), 1 µg template DNA (Qiagen-purified plasmid DNA from cDNA library pool #33) and 200 µM each dNTP using a DNA thermal cycler and 2.5 units of Taq polymerase (Perkin-Elmer, Cetus). Thirty cycles of PCR were performed using a cycle profile of denaturation at 94°C for 1 minute, annealing at 55°C for 2 minutes, and extension at 72°C for 3 minutes.

[0084] Twenty µl aliquots of the amplification products were analyzed by electrophoresis on 0.7% agarose gels revealing a 0.9 kb major product with one primer pair (sense, RGAE/s_2; antisense, pYES 2.0 reverse primer #43). The DNA fragment of interest was excised from the gel, recovered by electroelution (Sambrook *et al.* 1989) using type D-0405 seamless dialysis tubing (Sigma), followed by phenol extraction and ethanol precipitation at -20°C for 12 hours. The PCR product was blunt-ended at 37°C for 10 minutes in 20 µl buffer (20 mM Tris-acetate, pH 7.9, 10 mM MgAc, 50 mM KAc, 1 mM DTT) containing 50 µM each dNTP and 3 units of T4 DNA polymerase (New England Biolabs). The reaction was stopped by incubation at 70°C for 5 minutes, chilled on ice for 5 minutes and diluted in 50 µl of kinase buffer (70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT) followed by phosphorylation at 37°C for 30 minutes with T4 polynucleotide kinase (10 U, New England Biolabs) and 1 mM ATP pH 7.0 (Pharmacia), phenol extraction, ethanol precipitation and ligation at 16°C for 12 hours into Sma I -cut, dephosphorylated pUC18 vector (50 ng per ligation, Pharmacia).

[0085] *E. coli* DH5α was transformed to ampicillin resistance (Hanahan 1985) using 5 µl of the ligation mixture, and 13 clones were analyzed by isolation of plasmid miniprep. DNA (Sambrook *et al.* 1989), and EcoRI/HindIII digestion of the plasmid subclones, followed by sequencing the ends of the 0.9 kb insert from one subclone (pRGA19) with universal pUC primers (Sanger *et al.* 1977). Nucleotide sequence analysis of the pRGA19 subclone revealed a unique open reading frame which, in addition to the primer encoded amino acids, contained 10 additional residues concurring with the available NH_2-terminal sequence from the purified enzyme, thus confirming that the PCR had specifically amplified the desired region of the rhamnogalacturonan acetylesterase I cDNA (Fig. 8). Fig. 8 shows the nucleotide sequence of the 5'-end of the rga1 cDNA, and the deduced primary structure of RGAE I from *A. aculeatus.* The NH_2-terminal signal peptide preceding the mature protein is underlined.

Southern blot analysis

[0086] Qiagen purified DNA (3 µg) from eight individual cDNA library pools (#33, 90, 100, 130, 136, 140, 142, 148) was digested to completion with Eag I (3 U/µg DNA, New England Biolabs), fractionated on a 0.7% agarose gel, denatured and blotted to a nylon filter (Hybond-N, Amersham) using 10 x SSC (Sambrook et al. 1989) as transfer buffer (Southern 1975). The purified 0.9 kb RGAE I PCR fragment was ^{32}P-labeled (> 1 x 10^9 cpm/µg) by random-priming (Feinberg & Vogelstein 1983) and used as a probe in Southern analysis. The hybridization was carried out in 2 x SSC (Sambrook et al. 1989), 5 x Denhardt's solution (Sambrook et al. 1989), 1% (w/v) SDS and 100 µg/ml denatured salmon sperm DNA with a probe concentration of 2.5 ng/ml for 16 hours at 65°C followed by washes in 2 x SSC (2 x 15 minutes), 2 x SSC, 1% SDS at 65°C for 30 minutes, then in 0.2 x SSC, 1% SDS at 65°C for 30 minutes, and finally in 2 x SSC (2 x 15 minutes). The filter was autoradiographed at -80°C for 12 hours revealing a single strongly hybridising 1.0 kb fragment in each cDNA pool. This indicated that each analyzed pool contained a full-length cDNA copy encoding RGAE I from *A. aculeatus*. Therefore, pool #33 was chosen for further experiments.

Northern blot analysis

[0087] To estimate the steady-state levels of the *A. aculeatus* RGAE I mRNA during growth in the RS3 medium, poly (A)$^+$ RNA was isolated from mycelia harvested daily after 1 - 5 days growth in the above medium, and subjected to Northern analysis. The PolyA$^+$ RNAs (5 µg/sample) were electrophoresed in 1.2 agarose-2.2 M formaldehyde gels (Sambrook *et al.*, 1989) and blotted to a nylon membrane (Hybond-N, Amersham) with 10 x SSC (Sambrook *et al.*, 1989) as transfer buffer. The RNA ladder from Bethesda Research Laboratories was used as a size marker, and a random-primed (Feinberg & Vogelstein, 1983) ^{32}P-labeled 0.9 kb RGAE I PCR product (see previous section) as a probe in the Northern analysis. The hybridization was carried out in 5 x SSC (Sambrook *et al.*, 1989), 5 x Denhardt's solution (Sambrook *et al.*, 1989), 0.5% SDS (w/v) and 100 µg/ml denatured salmon sperm DNA with a probe concentration of ca. 2.5 ng/ml for 16 hours at 65°C followed by washes in 5 x SSC at 65°C (2 x 15 minutes), 2 x SSC, 0.5% SDS (1 x 30 minutes), 0.2 x SSC, 0.5% SDS (1 x 30 minutes), and 5 x SSC (2 x 15 minutes). The filter was autoradiographed at - 80°C for 4 days.

[0088] The RGAE I cDNA probe detects a 1.0 kb mRNA species readily in 5-day-old mycelia, and weakly in 4-day-old mycelia but not in 1 to 3-day-old mycelia coinciding with the decreasing levels of glucose in the growth medium after day 1, with no detectable glucose in the supernatant after day 3.

Isolation and characterization of a full-length cDNA encoding rhamnogalacturonan acetylesterase I (RGAE I) from A. aculeatus

[0089] To isolate a full-length cDNA clone for RGAE I, 30 000 colonies from the cDNA library pool #33 were plated on LB-agar (24 x 24 cm plate, Nunc) containing ampiciltin (100µg/ml) and replicated on an LB + amp - plate covered with a nylon filter (Hybond-N, Amersham). The purified 0.9 kb RGAE I PCR fragment was ^{32}P-labeled by random-priming as above, and used as a probe in screening the library pool by colony hybridization (Sambrook *et al.*, 1989). The hybridization was carried out in 2 x SSC (Sambrook *et al.* , 1989), 5 x Denhardt's solution (Sambrook *et al.* 1989), 1% (w/v) SDS and 100 µg/ml denatured salmon sperm DNA with a probe concentration of 2.5 ng/ml for 16 hours at 65°C, then in 2 x SSC (2 x 15 minutes), 0.2 x SSC, 1% SDS (2 x 30 minutes), and in 2 x SSC (2 x 15 minutes) followed by autoradiography at - 80°C for 12 hours. Screening of 30,000 colonies from pool 33 yielded 5 putative RGAE I cDNA clones that were colony-purified by two more rounds of hybridizations. One of these clones (designated pRGA1) was characterized by digesting the plasmid with HindIII and XbaI and sequencing the ends of the 1.0 kb cDNA insert with forward and reverse pYES 2.0 polylinker primers.

[0090] The 1.0 kb insert in pRGA1 contains a 0.85 kb open reading frame (ORF) initiating with an ATG codon at nucleotide position 40 and terminating with a TGA stop codon (Figs. 8 and 9). Fig. 9 shows the nucleotide sequence of the 3'-end of the rga1 cDNA and the deduced primary structure of RGAE I from *A. aculeatus*. The ORF is preceded by a 39 bp 5' non-coding region and followed by a 132 bp 3' non-coding region and a poly(A) tail. Comparison of the deduced protein sequence with the NH_2-terminal sequence of the purified mature RGAE I reveals that the cDNA encodes a precursor protein containing a 18 residue signal peptide (Fig. 8).

Construction of an *Aspergillus* expression vector

[0091] The vector pHD414 (Fig. 10) is a derivative of the plasmid p775 (described in EP 238 023). In contrast to this plasmid, pHD 414 has a string of unique restriction sites between the promoter and the terminator. The plasmid was constructed by removal of an approximately 200 bp long fragment (containing undesirable RE sites) at the 3'end of the terminator, and subsequent removal of an approximately 250 bp long fragment at the 5'end of the promoter, also

containing undesirable sites. The 200 bp region was removed by cleavage with NarI (positioned in the pUC vector) and XbaI (just 3' to the terminator), subsequent filling in the generated ends with Klenow DNA polymerase +dNTP, purification of the vector fragment on gel and religation of the vector fragment. This plasmid was called pHD413. pHD413 was cut with StuI (positioned in the 5'end of the promoter) and PvuII (in the pUC vector), fractionated on gel and religated, resulting in pHD414. Fig. 10 is a map of plasmid pHD414, wherein "AMG Terminator" indicates the *A. niger* glucoamylase terminator, and "TAKA Promoter" indicates the *A. oryzae* TAKA amylase promoter. pHD464 is a derivative of pHD414, in which the 5' non-translated region is substituted by the 5' non-translated region from the *Aspergillus TPi* gene.

Construction of the RGAE I expression cassette

[0092] Plasmid miniprep. DNA from pRGA1 was digested with BamHI and XhoI, electrophoresed in 0.7% agarose gel, followed by purification of the 1.0 kb cDNA insert using the Geneclean II kit according to the manufacturer's instructions (Bio 101 Inc., La Jolla) and ligation into BamHI/XhoI-cleaved pHD414 vector. One µl of the ligation mixture was electroporated into *E. coli* 1061 and two transformants analyzed by BamHI/XhoI digestion of plasmid miniprep. DNA. Since both clones (designated pRGA1 and pRGA2) contained a correct sized insert, pRGA1 was chosen for further experiments. A midi preparation of the pRGA1 expression plasmid (Qiagen Tip 100, see section 4) was checked by sequencing the 5'-end of the construct and used in *Aspergillus oryzae* transformation.

Transformation of Aspergillus oryzae and analysis of the transformants

[0093] 100 ml of the Aspergillus minimal medium (1 M sucrose, 10 mM urea, 0.52 mg/ml KCl, 0.52 mg/ml $MgSO_4$, 1.52 mg/ml KH_2PO_4, 0.04 µg/ml $Na_2B_4O_7$, 0.4 µg/ml $CuSO_4$, 0.8 µg/ml $FePO_4$, 0.8µg/ml $MnSO_4$. 0.8 µg/ml Na_2MoO_4, 8 µg/ml $ZnSO_4$) was inoculated with spore suspensions from *A. oryzae* strains 1560 or 1560-710 or *A. niger*, the mycelium was collected by filtration through sterile Miracloth after 24 hours growth at 30°C, washed with 200 ml 0.6 M $MgSO_4$, suspended in 25 ml of cold 1.2 M $MgSO_4$, 10 mM NaH_2PO_4, pH 5.8 containing 2 mg/ml Novozym® (Novo Nordisk A/S) and incubated on ice for 5 minutes. One ml of BSA (12 mg/ml. sterile filtered) was added to the mycelia and the suspension was incubated at 37°C for 1.5 hour with gentle shaking. Protoplasts were separated from undigested mycelia debris by filtering through Miracloth, overlayed with 5 ml 0.6 M sorbitol, 100 mM Tris-HCl, pH 7.0, and centrifuged at 2500 rpm for 15 minutes. Protoplasts were collected from interphase, washed four times in 3 ml of 1.2 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, resuspended in the same buffer at a concentration of between 5 x 10^7-5 x 10^8/ml and used immediately.

[0094] Transformation of *A. oryzae* or *A. niger* was carried out essentially as follows. Eight µg of Qiagen purified pRGA1 plasmid DNA (1 µg/µl) was mixed with 1 µg of Qiagen purified ToC 90 (1µg/µl) co-plasmid DNA, an *A. nidulans* AmdS gene carrying plasmid, and 100 µl of protoplast suspension, and incubated at RT for 20 minutes. 250µl of 60% (w/v) PEG, 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$ was added and the mixture incubated at RT for 20 minutes. After addition of 3 ml 1.2 M sorbitol, the transformation mixture was centrifuged at 2500 rpm for 10 minutes, resuspended in 100 µl 1.2 M sorbitol, spread on AmdS selection plates and incubated at 30°C for 3 to 5 days.

[0095] Twenty ml aliquots of YP + maltodextrin (2%) medium were inoculated with spore suspensions from AmdS+ transformants cotransformed with the ToC plasmid (4 in A. oryzae 1560-710, 2 in the strain 1560), followed by growth at 30°C for 2 to 3 days. The transformants were screened for RGAE I activity by assaying the culture medium for rhamnogalacturonan acetylesterase and acetylesterase activities. The amount and purity of proteins secreted to the culture medium in various transformants was evaluated by analyzing 4 µl aliquots of the supernatant fractions on 10% SDS-PAGE (Fey *et al.* 1984) followed by either Coomassie or silver stain, using the purified RGAE I enzyme preparation from *A. aculeatus* as a control.

Enzymatic assays

[0096] The acetylesterase activity in the culture medium of the AmdS+ transformants was measured as increase of absorbance at OD_{405} after incubation of 20 µl samples at 40°C for 30 minutes in 500 µl of 2 mM p-nitrophenyl-acetate/ 20 mM Na-citrate buffer; pH 5.0 using culture medium from *A. oryzae* 1560-710 as a negative control and the purified RGAE I preparation (0.78 mg/ml) as a positive control. Before measurement, the pH was raised by adding 1 ml of Tris-HCl, pH 7.0 to the samples. Due to a high acetylesterase activity in the control strain all samples exhibited a comparable activity towards p-nitrophenyl-acetate (data not shown) with no detectable increase in the AmdS+ transformants.

[0097] The rhamnogalacturonan acetylesterase activity in the culture medium (see above) was measured as a release of acetate from modified hairy regions (MHR) isolated from apple pectin (Schols *et al.* 1990). The samples (50 µl of each supernatant) were incubated with 100 µl of 1% (w/v, in H_2O) modified hairy regions for a) 2 hours and b) 24

hours using the same control samples as above. The determination of acetic acid was carried out using the acetic acid kit from Boehringer Mannheim according to the manufacturer's instructions. Five out of six AmdS+ transformants show a clear activity towards 1% MHR , while the sixth transformant exhibit no activity compared to the *A. oryzae* control strain 1560-710 (Fig. 11). Fig. 11 shows the activity towards 1% modified hairy regions (MHR), produced by recombinant *A. oryzae* strains expressing rhamnogalacturonan acetyl esterase I from *A. aculeatus*. The enzymatic activities were measured as a release of acetate from apple pectin MHR. The highest activities observed in the transformants RGAE 710-1 and RGAE 710-4 coincide with a ca. 35 kDa protein secreted into the culture medium. The protein is slightly overglycosylated compared to the purified RGAE I from *A. aculeatus* as judged by SDS-PAGE.

Media

[0098]

YPD: 10 g yeast extract, 20 g peptone, H_2O to 810 ml. Autoclaved, 90 ml 20% glucose (sterile filtered) added.

YPG-agar: 25 g/l Bactoagar, 15 g/l glucose, 5 g/l K_2PO_4, 0.5 g/l $MgSO_4$-$7H_2O$, pH adjusted to 5.0. Autoclaved.

10 x Basal salt: 66.8 g yeast nitrogen base, 100 g succinic acid, 60 g NaOH, H_2O ad 1000 ml, sterile filtered.

SC-URA: 90 ml 10 x Basal salt, 22.5 ml 20% casamino acids, 9 ml 1% tryptophane, H_2O ad 806 ml, autoclaved, 3.6 ml 5% threonine and 90 ml 20% glucose added.

SC-H agar: 7.5 g/l yeast nitrogen base without amino acids, 11.3 g/l succinic acid, 6.8 g/l NaOH, 5.6 g/l casamino acids without vitamins, 0.1 g/l tryptophan and 20 g/l agar (Bacto). Autoclaved for 20 minutes at 121°C. After autoclaving, 55 ml of a 22% galactose solution and 1.8 ml of a 5% threonine solution were added per 450 ml agar.

YNB-1 agar: 3.3 g/l KH_2PO_4, 16.7 g/l agar, pH adjusted to 7. Autoclaved for 20 minutes at 121°C. After autoclaving, 25 ml of a 13.6% yeast nitrogen base without amino acids, 25 ml of a 40% glucose solution, 1.5 ml of a 1% L-leucine solution and 1.5 ml of a 1% histidine solution were added per 450 ml agar.

YNB-1 broth: Composition as YNB-1 agar, but without the agar.

MHR overlayer gel: 1% agarose, 0,5% MHR in 0,05 M Na-acetate buffer, pH 4.5. The gel was boiled and then cooled to 55°C before the overlayer was poured onto agar plates.

FG-4-Agar: 35 g/l agar, 30 g/l Soy bean meal, 15 g/l maltodextrin (Glucidex 6), 5 g/l Bacto pepton, pH 7. Autoclaved 40 minutes at 121°C

FG-4 medium: 30 g/l Soy bean meal, 15 g/l maltodextrin (Glucidex 6), 5 g/l Bacto pepton. Autoclaved 40 minutes at 121°C.

MDU-2 medium: 45 g/l maltose, l g/l $MgSO_4$ - 7 H_2O, 1 g/l NaCl, 2g/l K_2SO_4, 12 g/l KH_2PO_4, 0.1 ml/l Pluronic 61 L, 0.5 ml/l Trace metal solution. pH 5.0. Autoclaved 20 minutes at 121°C. 15 ml/l 50% Sterile filtered urea is added after autoclavation.

Trace metal solution: 13.9 g/l $FeSO_4$-7 H_2O, 8.45 g/l $MnSO_4$-H_2O, 6.8 g/l $ZnCl_2$, 2.5 g/l $CuSO_4$-$5H_2O$, 0.24 g/l $NiCl_2$-$6H_2O$, 3 g/l citric acid.

[0099] For a better understanding of the invention reference is made to the following references.

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SEQUENCE LISTING

[0100]

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-2880
(G) TELEPHONE: +45 4444 8888
(H) TELEFAX: +45 4449 3256
(I) TELEX: 37304

(ii) TITLE OF INVENTION: A Novel Enzyme and a DNA Sequence

(iii) NUMBER OF SEQUENCES: 10

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EP0)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Aspergillus aculeatus
(B) STRAIN: CBS 101.43

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Asp Arg Val Tyr Leu Ala Gly Asp Ser Thr Met Thr Lys Asn Gly Gly
1               5                  10                  15

Xaa Ser Gly Thr Asn Gly Trp Gly Glu Tyr Leu Ala
            20                  25
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ATGAAGACCG CCGCCTCTTG CACCGCTCTT CTTCCTCCCC TCTGCCCTCG CCACGACNNG 60

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GTCTATCTCG CGGGTGACTC GACCATGGCC AAGAATGGAG GCGGGTCGGG AACTAACGGC 60

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TGGGGCGAGT ACCTGCGAGT TACCTCTCCG CGACAGTGGT TAACGACGCG GTCGCG 56

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY; linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GACGCAACCT ATGAAGACCT TGGAATGCCA CCGTCAACTC GTATTCCCCA TCGATCACAC 60

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CCACACCAGT CCTGCGGCGC GAGGTCGTGG CTGAGCGTTC TTGAAGGCGG TGGTATGCAC 60

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GGGTACGTCG TTGAAGAGTG TGTTGACGAC GACGAGCTT 39

(2) INFORMATION FOR SEQ ID NO: 8:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 176 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

ATGAAGACCG CCGCCTCTTG CACCGCTCTT CTTCCTCCCC TCTGCCCTCG CCACGACNNG 60

GTCTATCTCG CGGGTGACTC GACCATGGCC AAGAATGGAG GCGGGTCGGG AACTAACGGC 120

TGGGGCGAGT ACCTGCGAGT TACCTCTCCG CGACAGTGGT TAACGACGCG GTCGCG 176

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 159 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GACGCAACCT ATGAAGACCT TGGAATGCCA CCGTCAACTC GTATTCCCCA TCGATCACAC 60

CCACACCAGT CCTGCGGCGC GAGGTCGTGG CTGAGCGTTC TTGAAGGCGG TGGTATGCAC 120

GGGTACGTCG TTGAAGAGTG TGTTGACGAC GACGAGCTT 159

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 935 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

```
AGTCTATAAG AAGATTGACA GCCAAGAACA CCACCCACAA TGAAGACCGC CGCCTCTTGC      60
ACCGCTCTTC TTCCTCCCCT CTGCCCTCGC CACGACNNGG TCTATCTCGC GGGTGACTCG     120
ACCATGGCCA AGAATGGAGG CGGGTCGGGA ACTAACGGCT GGGGCGAGTA CCTCGCCAGT     180
TACCTCTCCG CGACAGTGGT TAACGACGCG GTCGCGGGCC GCAGCGCGCG CTCGTACACA     240
CGCGAGGGTC GGTTCGAGAA CATCGCCGAT GTAGTGACGG CGGGCGACTA CGTGATCGTC     300
GAGTTCGGCC ACAACGACGG TGGCTCGCTG TCCACGGACA ATGGACGCAC CGACTGCTCC     360
GGTACCGGGG CAGAAGTCTG CTATAGCGTC TACGACGGGG TCAACGAGAC CATCCTCACC     420
TTCCCCGCCT ACCTGGAGAA CGCCGCCAAG CTGTTCACCG CCAAGGGCGC CAAGGTCATT     480
CTCAGCAGCC AGACCCCCAA CAACCCCTGG GAGACCGGCA CCTTCGTCAA CTCCCCCACG     540
CGCTTCGTTG AGTACGCCGA GCTGGCCGCC GAGGTCGCTG GCGTCGAGTA CGTCGACCAC     600
TGGTCCTACG TGGACAGCAT CTATGAGACC TTGGCAATGC CACCGTCAAC TCGTATTCCC     660
CATCGATCAC ACCCACACCA GTCCTGCGGC GCGAGGTCGT GGCTGAGCGT TCTTGAAGGC     720
GGTGGTATGC ACGGGTACGT CGTTGAAGAG TGTGTTGACG ACGACGAGCT TTGAGGGGAC     780
ATGTCTGTGA TTGAGCAGAT GGAAAGACAA AGGAGTGGAC GGATAAGGAC AGGAGTTGTC     840
ATGTATAGTG GTAGTTTGTG CATTGCAAAT GGTATCTGAA CTGGCTCGCT TATGCTCATG     900
ATCGACAAAA AAAAAAAAAA AAAAAAAAAA AAAAA                                 935
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Claims

1. A purified polypeptide having rhamnogalacturonan acetylesterase (RGAE) activity, which comprises the partial amino add sequence of SEQ ID No. 1, or a partial amino add sequence with a homology to SEQ ID No. 1 of at least 70%.

2. The polypeptide of claim 1 which comprises the partial amino add sequence of SEQ ID No. 1,

3. The polypeptide of claim 1 or 2 which is capable of releasing acetate from apple pectin modified hairy regions (MHR).

4. The polypeptide of any one of claims 1-3, wherein the RGAE activity is measured using the assay described at p. 28 of the description ("The rhamnogalacturonan acetylesterase activity — manufacturer's instructions").

5. The polypeptide of claim 4, wherein the RGAE activity corresponds to the release of at least about 12.5 µg acetic add/ml.

6. The polypeptide of any one of claims 1-5 which comprises at least one of

   a) the amino add sequence shown in Fig. 7;
   b) the amino add sequence shown in Fig. 8, or the sequence of amino adds 19-59 therein;

c) the amino acid sequence shown in Fig. 9;
d) the amino acid sequence which is encoded by the DNA sequence of SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7; or SEQ ID No. 8, SEQ ID No. 9; or SEQ ID No. 10;
e) an amino add sequence which is encoded by a DNA sequence that

i) hybridizes to the DNA sequence of SEQ ID No. 10 under the following conditions: presoaking in 5xSSC and prehybridizing for 1 hour at ~40°C in a solution of 5xSSC, 5xDenhardt's solution, 50 mM sodium phosphate, pH 6.8, and 50 µg of denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 50 mCi 32-P-dCTP labelled probe for 18 hours at ~40°C followed by washing three times in 2xSSC, 0.2% SDS at 40°C for 30 minutes;
ii) hybridizes to the DNA sequence of SEQ ID No. 10 under any of the conditions described at p. 24-25 of the description ("Southern blot analysis — and reverse pYES 2.0 polylinker primers.");
iii) is at least 70% homologous to SEQ ID No.10.

7. The polypeptide of any one of claims 1-6, which is immunologically reactive with an antibody raised against a purified RGAE derived from *Aspergillus aculeatus,* CBS 101.43.

8. A recombinant DNA sequence which comprises a DNA sequence that encodes the polypeptide of any one of claims 1-7.

9. The recombinant DNA sequence of claim 8, wherein the DNA sequence comprises at least one of

a) the DNA sequences shown in Fig. 7;
b) the DNA sequence shown in Fig. 8, or nucleotides 40-215 thereof;
c) the DNA sequence shown in Fig. 9; or nucleotides 1-159 thereof;
d) the following DNA sequences SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7; or SEQ ID No. 8, SEQ ID No.9; or SEQ ID No.10;
e) a DNA sequence that

i) hybridizes to the DNA sequence of SEQ ID No. 10 under the following conditions: presoaking in 5xSSC and prehybridizing for 1 hour at ~40°C in a solution of 5xSSC, 5xDenhardt's solution, 50 mM sodium phosphate, pH 6.8, and 50 µg of denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 50 mCi 32-P-dCTP labelled probe for 18 hours at ~40°C followed by washing three times in 2xSSC, 0.2% SDS at 40°C for 30 minutes;
ii) hybridizes to the DNA sequence of SEQ ID No. 10 under any of the conditions described at p. 24-25 of the description ("Southern blot analysis — and reverse pYES 2.0 polylinker primers.");
iii) is at least 70% homologous to SEQ ID No. 10.

10. A vector comprising the recombinant DNA sequence according to any one of claims 8-9

11. A transformed host cell containing the vector according to claim 10.

12. A method for production of a polypeptide having RGAE activity by use of a transformed host cell of claim 11.

13. Use of the polypeptide having RGAE activity according to any one of claims 1-7 for at least one of the following purposes

a) as an agent for degradation or modification of MHR or acetylated rhamnogalacturonan;
b) as an agent for degradation or modification of plant cell walls;
c) in combination with other plant cell wall degrading enzymes;
d) in combination with other plant cell wall degrading enzymes, to prepare a product of an increased proportion of RGAE activity;
e) in combination with a pectinase preparation usable for degradation or modification of plant cell walls;
f) in combination with a pectinase preparation usable for degradation or modification of plant cell walls, to prepare a product of an increased proportion of RGAE activity,
g) together with enzymes specific to deacetylated or partially deacetylated MHR;
h) together with enzymes specific to deacetylated or partially deacetylated MHR, to prepare a product of an increased proportion of RGAE activity.

Patentansprüche

1. Gereinigtes Polypeptid mit Rhamnogalacturonanacetylesterase-(RGAE)-Aktivität, das die teilweise Aminosäuresequenz von SEQ ID Nr. 1 oder eine teilweise Aminosäuresequenz mit einer Homologie von wenigstens 70% zu SEQ ID Nr. 1 umfasst.

2. Polypeptid nach Anspruch 1, das die teilweise Aminosäuresequenz von SEQ ID Nr. 1 umfasst.

3. Polypeptid nach Anspruch 1 oder 2, das die Fähigkeit besitzt, Acetat aus Apfelpektin-modifizierten haarigen ("hairy") Regionen (MHR) freizusetzen.

4. Polypeptid nach einem der Ansprüche 1-3, wobei die RGAE-Aktivität gemessen wird unter Verwendung des Assays, der auf S. 28 der Beschreibung ("Die Rhamnogalacturonanacetylesterase-Aktivität -- Anweisungen des Herstellers") beschrieben ist.

5. Polypeptid nach Anspruch 4, wobei die RGAE-Aktivität der Freisetzung von wenigstens ca. 12,5 µg Essigsäure/ml entspricht.

6. Polypeptid nach einem der Ansprüche 1-5, das umfasst wenigstens eine von

   a) die Aminosäuresequenz, die in Fig. 7 gezeigt ist;
   b) die Aminosäuresequenz, die in Fig. 8 gezeigt ist, oder die Sequenz von Aminosäuren 19-59 darin;
   c) die Aminosäuresequenz, die in Fig. 9 gezeigt ist;
   d) die Aminosäuresequenz, die kodiert wird von der DNA-Sequenz von SEQ ID Nr. 2, SEQ ID Nr. 3, SEQ ID Nr. 4, SEQ ID Nr. 5, SEQ ID Nr. 6, SEQ ID Nr. 7; oder SEQ ID Nr. 8, SEQ ID Nr. 9; oder SEQ ID Nr. 10;
   e) eine Aminosäuresequenz, die von einer DNA-Sequenz kodiert wird, die

      i) mit der DNA-Sequenz von SEQ ID Nr. 10 unter den folgenden Bedingungen hybridisiert: Prä-Tränken in 5xSSC und Prähybridisieren für 1 Stunde bei ~40°C in einer Lösung aus 5xSSC, 5xDenhardt's Lösung, 50 mM Natriumphosphat, pH 6,8, und 50 µg denaturierter sonifizierter Kalbsthymus-DNA, gefolgt von Hybridisieren in derselben Lösung, die ergänzt ist mit 50 mCi 32-P-dCTPmarkierter Sonde für 18 Stunden bei ~40°C gefolgt von drei Waschzeiten in 2xSSC, 0,2% SDS bei 40°C für 30 Minuten;
      ii) mit der DNA-Sequenz von SEQ ID Nr. 10 unter irgendeiner der Bedingungen hybridisiert, die auf S. 24-25 der Beschreibung ("Southern-Blot-Analyse-- und reverse pYES 2,0 Polylinker-Primer.") beschrieben sind;
      iii) wenigstens 70% homolog zu SEQ ID Nr. 10 ist.

7. Polypeptid nach einem der Ansprüche 1-6, das immunologisch reaktiv ist mit einem Antikörper, der gegen eine gereinigte RGAE abgeleitet von *Aspergillus aculeatus*, CBS 101.43, gerichtet ist.

8. Rekombinante DNA-Sequenz, die eine DNA-Sequenz umfasst, die das Polypeptid nach einem der Ansprüche 1-7 kodiert.

9. Rekombinante DNA-Sequenz nach Anspruch 8, wobei die DNA-Sequenz umfasst wenigstens eine von

   a) die DNA-Sequenzen, die in Fig. 7 gezeigt sind;
   b) die DNA-Sequenz, die in Fig. 8 gezeigt ist; oder Nukleotide 40-215 davon;
   c) die DNA-Sequenz, die in Fig. 9 gezeigt ist; oder Nukleotide 1-159 davon;
   d) die folgenden DNA-Sequenzen SEQ ID Nr. 2, SEQ ID Nr. 3, SEQ ID Nr. 4, SEQ ID Nr. 5, SEQ ID Nr. 6, SEQ ID Nr. 7; oder SEQ ID Nr. 8, SEQ ID Nr. 9; oder SEQ ID Nr. 10;
   e) eine DNA-Sequenz, die

      i) mit der DNA-Sequenz von SEQ ID Nr. 10 unter den folgenden Bedingungen hybridisiert: Prä-Tränken in 5xSSC und Prähybridisieren für 1 Stunde bei ~40°C in einer Lösung aus 5xSSC, 5xDenhardt's Lösung, 50 mM Natriumphosphat, pH 6,8, und 50 µg denaturierter sonifizierter Kalbsthymus-DNA, gefolgt von Hybridisieren in derselben Lösung, die ergänzt ist mit 50 mCi 32-P-dCTPmarkierter Sonde für 18 Stunden bei ~40°C gefolgt von drei Waschzeiten in 2xSSC, 0,2% SDS bei 40°C für 30 Minuten;
      ii) mit der DNA-Sequenz von SEQ ID Nr. 10 unter irgendeiner der Bedingungen hybridisiert, die auf S.

24-25 der Beschreibung ("Southern-Blot-Analyse-- und reverse pYES 2,0 Polylinker-Primer.") beschrieben sind;
iii) wenigstens 70% homolog zu SEQ ID Nr. 10 ist.

10. Vektor umfassend die rekombinante DNA-Sequenz gemäß einem der Ansprüche 8-9.

11. Transformierte Wirtszelle enthaltend den Vektor gemäß Anspruch 10.

12. Verfahren zur Herstellung eines Polypeptids mit RGAE-Aktivität unter Verwendung einer transformierten Wirtszelle nach Anspruch 11.

13. Verwendung des Polypeptids mit RGAE-Aktivität gemäß einem der Ansprüche 1-7 für wenigstens einen der folgenden Zwecke

a) als ein Mittel zur Degradation oder Modifikation von MHR oder acetyliertem Rhamnogalacturonan;
b) als ein Mittel zur Degradation oder Modifikation von Pflanzenzellwänden;
c) in Kombination mit anderen Pflanzenzellwand-degradierenden Enzymen;
d) in Kombination mit anderen Pflanzenzellwand-degradierenden Enzymen, um ein Produkt mit einem gesteigerten Anteil an RGAE-Aktivität zuzubereiten;
e) in Kombination mit einer Pektinase-Zubereitung verwendbar zur Degradation oder Modifikation von Pflanzenzellwänden;
f) in Kombination mit einer Pektinase-Zubereitung verwendbar zur Degradation oder Modifikation von Pflanzenzellwänden, um ein Produkt mit einem gesteigerten Anteil an RGAE-Aktivität zuzubereiten;
g) zusammen mit Enzymen, die spezifisch fiir deacetylierte oder teilweise deacetylierte MHR sind;
h) zusammen mit Enzymen, die spezifisch sind fiir deacetylierte oder teilweise deacetylierte MHR, um ein Produkt mit einem gesteigerten Anteil an RGAE-Aktivität zuzubereiten.

Revendications

1. Polypeptide purifié ayant une activité rhamnogalacturonan acétylestérase (RGAE), qui comprend la séquence d'acides aminés partielle de SEQ ID No. 1, ou une séquence d'acides aminés partielle avec une homologie à SEQ ID No. 1 d'au moins 70%.

2. Polypeptide de la revendication 1 qui comprend la séquence d'acides aminés partielle de SEQ ID No. 1.

3. Polypeptide de la revendication 1 ou 2 qui est capable de libérer de l'acétate à partir de régions chevelues modifiées (MHR) de pectine de pomme.

4. Polypeptide de l'une quelconque des revendications 1-3, dans lequel l'activité RGAE est mesurée en utilisant le dosage décrit à la page 29 de la description ("L'activité rhamnogalacturonan acétylestérase ... instructions du fabricant").

5. Polypeptide de la revendication 4, dans lequel l'activité RGAE correspond à la libération d'au moins environ 12,5 µg d'acide acétique/ml.

6. Polypeptide de l'une quelconque des revendications 1-5 qui comprend au moins une de

a) la séquence d'acides aminés présentée sur la figure 7 ;
b) la séquence d'acides aminés présentée sur la figure 8, ou la séquence des acides aminés 19-59 à l'intérieur de celle-ci ;
c) la séquence d'acides aminés présentée sur la figure 9 ;
d) la séquence d'acides aminés qui est codée par la séquence d'ADN de SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7 ; ou SEQ ID No. 8, SEQ ID No. 9; ou SEQ ID No. 10 ;
e) une séquence d'acides aminés qui est codée par une séquence d'ADN qui

(i) s'hybride avec la séquence d'ADN de SEQ ID No. 10 dans les conditions suivantes : pré-trempage dans SSC 5x et pré-hybridation pendant 1 heure à ~ 40°C dans une solution de SSC 5x, solution de

Denhardt 5x, phosphate de sodium 50 mM, pH 6,8, et 50 µg d'ADN de thymus de veau ultrasoniqué dénaturé, suivi d'une hybridation dans la même solution complétée par une sonde marquée à 32-P-dCTP 50 mCi pendant 18 heures à ~ 40°C suivi d'un lavage par trois fois dans SSC 2x, SDS à 0,2% à 40°C pendant 30 minutes ;

(ii) s'hybride avec la séquence d'ADN de SEQ ID No. 10 dans l'une quelconque des conditions décrites aux pages 24-26 de la description ("Analyse Southern Blot ... amorces avant et inverse de lieur multisite pYES 2.0.");

(iii) est homologue à au moins 70% à SEQ ID No. 10.

7. Polypeptide de l'une quelconque des revendications 1-6, qui est immunologiquement réactif à un anticorps dressé contre une RGAE purifiée issue d'*Aspergillus aculeatus*, CBS 101.43.

8. Séquence d'ADN recombinant qui comprend une séquence d'ADN qui code le polypeptide de l'une quelconque des revendications 1-7.

9. Séquence d'ADN recombinant de la revendication 8, dans laquelle la séquence d'ADN comprend au moins une de

a) les séquences d'ADN présentées sur la figure 7 ;
b) la séquence d'ADN présentée sur la figure 8, ou les nucléotides 40-215 de celle-ci ;
c) la séquence d'ADN présentée sur la figure 9, ou les nucléotides 1-159 de celle-ci ;
d) les séquences d'ADN suivantes SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7; ou SEQ ID No. 8, SEQ ID No. 9; ou SEQ ID No. 10;
e) une séquence d'ADN qui

(i) s'hybride avec la séquence d'ADN de SEQ ID No. 10 dans les conditions suivantes : pré-trempage dans SSC 5x et pré-hybridation pendant 1 heure à ~ 40°C dans une solution de SSC 5x, solution de Denhardt 5x, phosphate de sodium 50 mM, pH 6,8, et 50 µg d'ADN de thymus de veau ultrasoniqué dénaturé, suivi d'une hybridation dans la même solution complétée par une sonde marquée à 32-P-dCTP 50 mCi pendant 18 heures à ~ 40°C suivi d'un lavage par trois fois dans SSC 2x, SDS à 0,2% à 40°C pendant 30 minutes ;

(ii) s'hybride avec la séquence d'ADN de SEQ ID No. 10 dans l'une quelconque des conditions décrites aux pages 24-26 de la description ("Analyse Southern Blot ... amorces avant et inverse de lieur multisite pYES 2.0.");

(iii) est homologue à au moins 70% à SEQ ID No. 10.

10. Vecteur comprenant la séquence d'ADN recombinant selon l'une quelconque des revendications 8-9.

11. Cellule hôte transformée contenant le vecteur selon la revendication 10.

12. Procédé de production d'un polypeptide ayant une activité RGAE par utilisation d'une cellule hôte transformée de la revendication 11.

13. Utilisation du polypeptide ayant une activité RGAE selon l'une quelconque des revendications 1-7 pour au moins un des usages suivants

a) comme agent de dégradation ou de modification de MHR ou de rhamnogalacturonan acétylé ;
b) comme agent de dégradation ou de modification de parois cellulaires végétales ;
c) en combinaison avec d'autres enzymes dégradant des parois cellulaires végétales ;
d) en combinaison avec d'autres enzymes dégradant des parois cellulaires végétales, pour préparer un produit avec une proportion accrue d'activité RGAE;
e) en combinaison avec une préparation de pectinase utilisable pour la dégradation ou la modification de parois cellulaires végétales ;
f) en combinaison avec une préparation de pectinase utilisable pour la dégradation ou la modification de parois cellulaires végétales, pour préparer un produit avec une proportion accrue d'activité RGAE ;
g) conjointement avec des enzymes spécifiques à MHR désacétylé ou partiellement désacétylé;
h) conjointement avec des enzymes spécifiques à MHR désacétylé ou partiellement désacétylé, pour préparer un produit avec une proportion accrue d'activité RGAE.

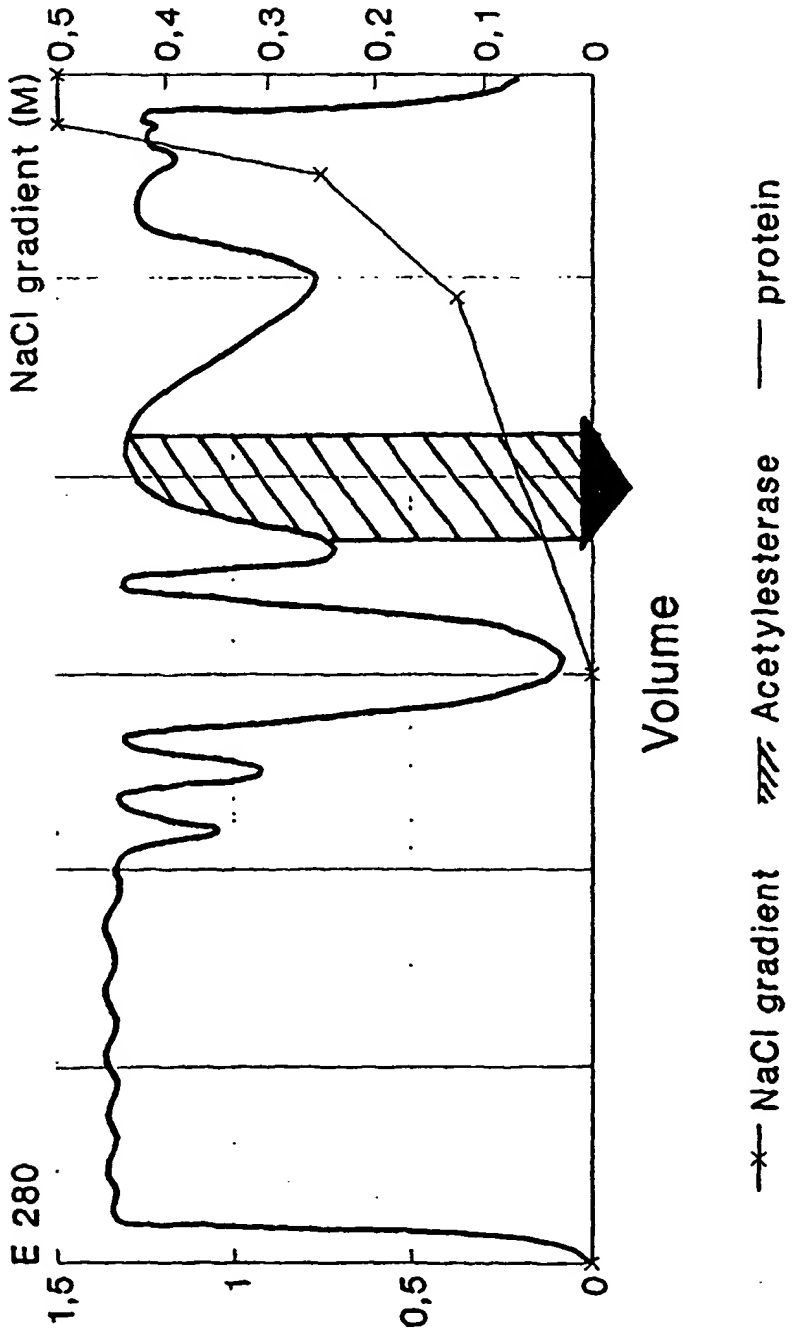
NaCl gradient (M)
0,5
0,4
0,3
0,2
0,1
0
E 280
1,5
1
0,5
0
Volume
NaCl gradient
Acetylesterase
protein

FIG. 1

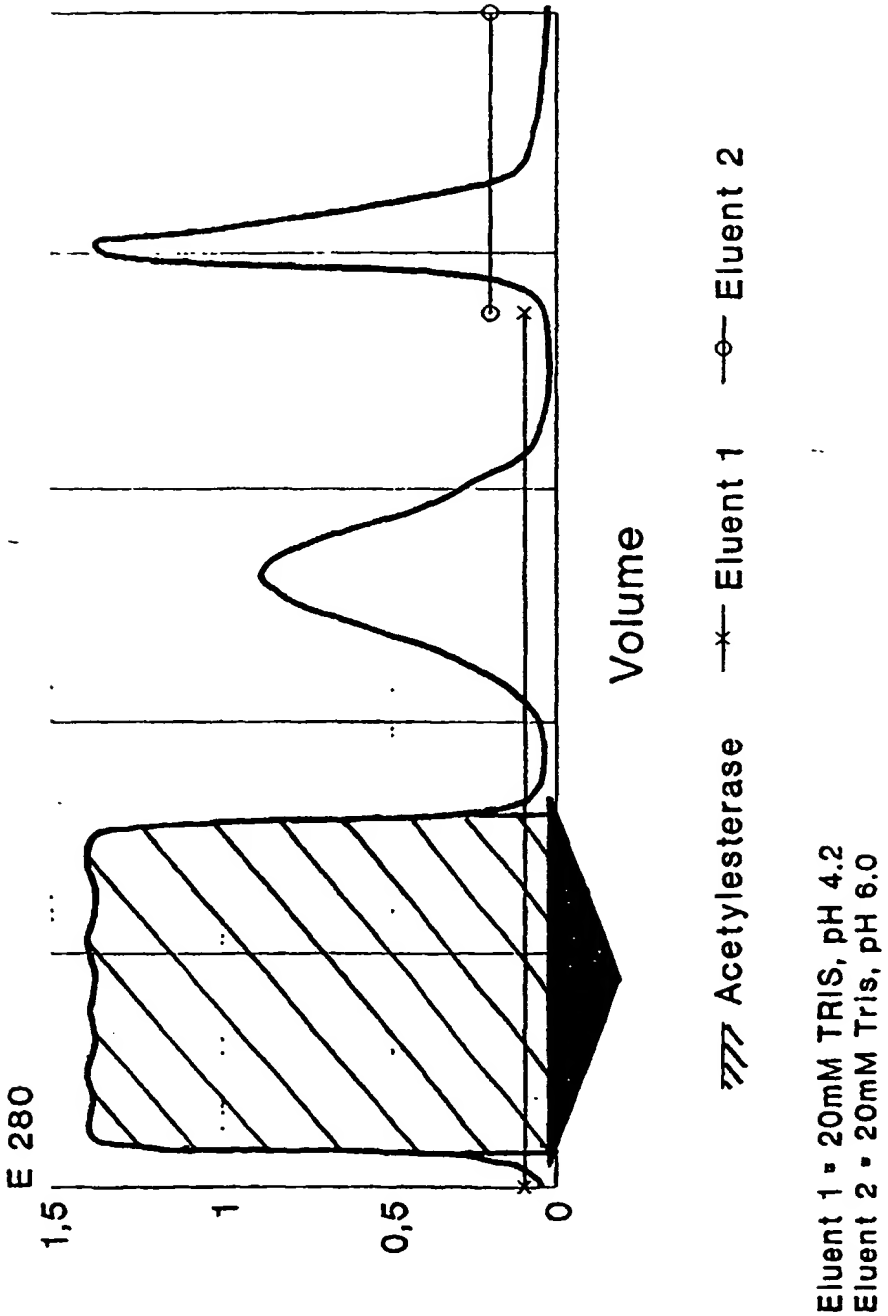
E 280
1,5
1
0,5
0
Volume
Acetylesterase
Eluent 1
Eluent 2
Eluent 1 = 20mM TRIS, pH 4.2
Eluent 2 = 20mM Tris, pH 6.0

FIG. 2

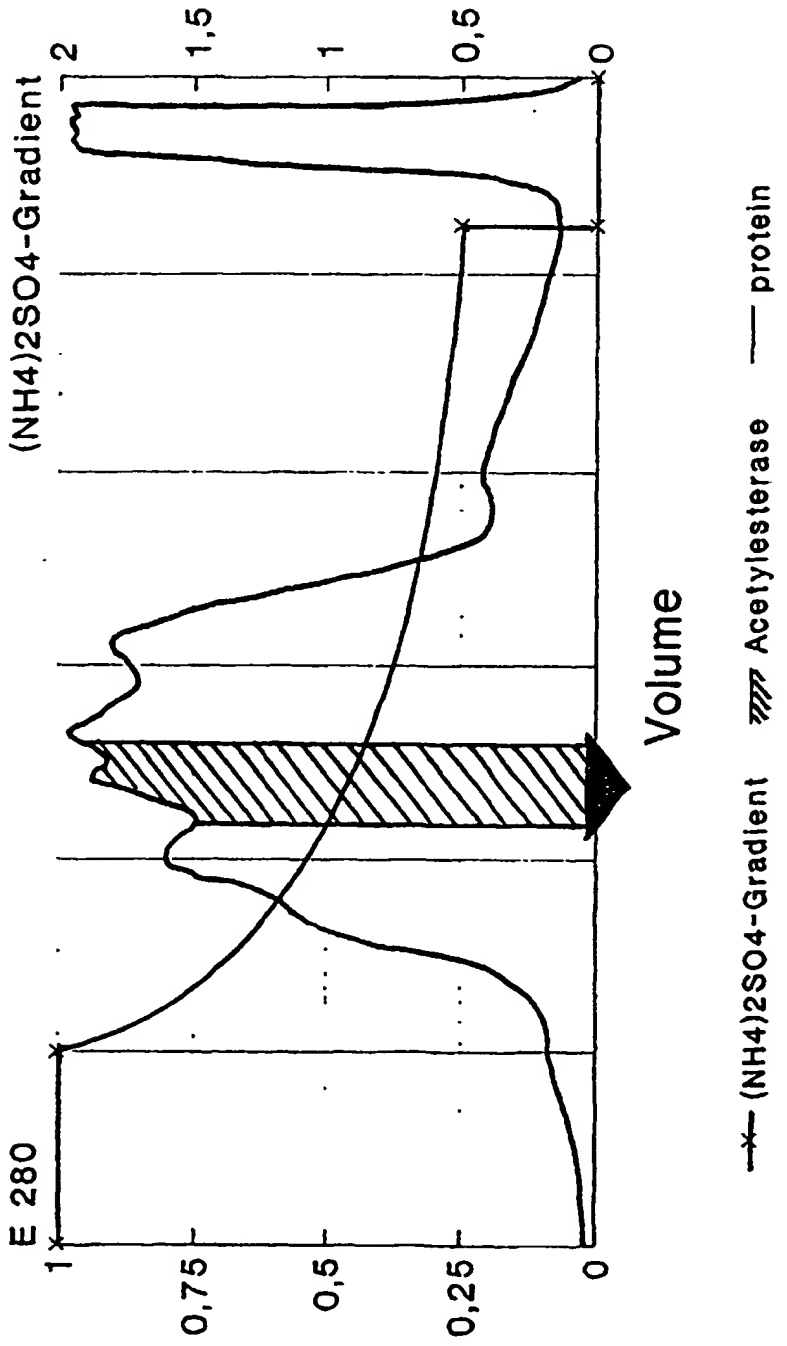
(NH4)2SO4-Gradient
2
1,5
1
0,5
0
E 280
1
0,75
0,5
0,25
0
Volume
(NH4)2SO4-Gradient
Acetylesterase
protein

FIG. 3

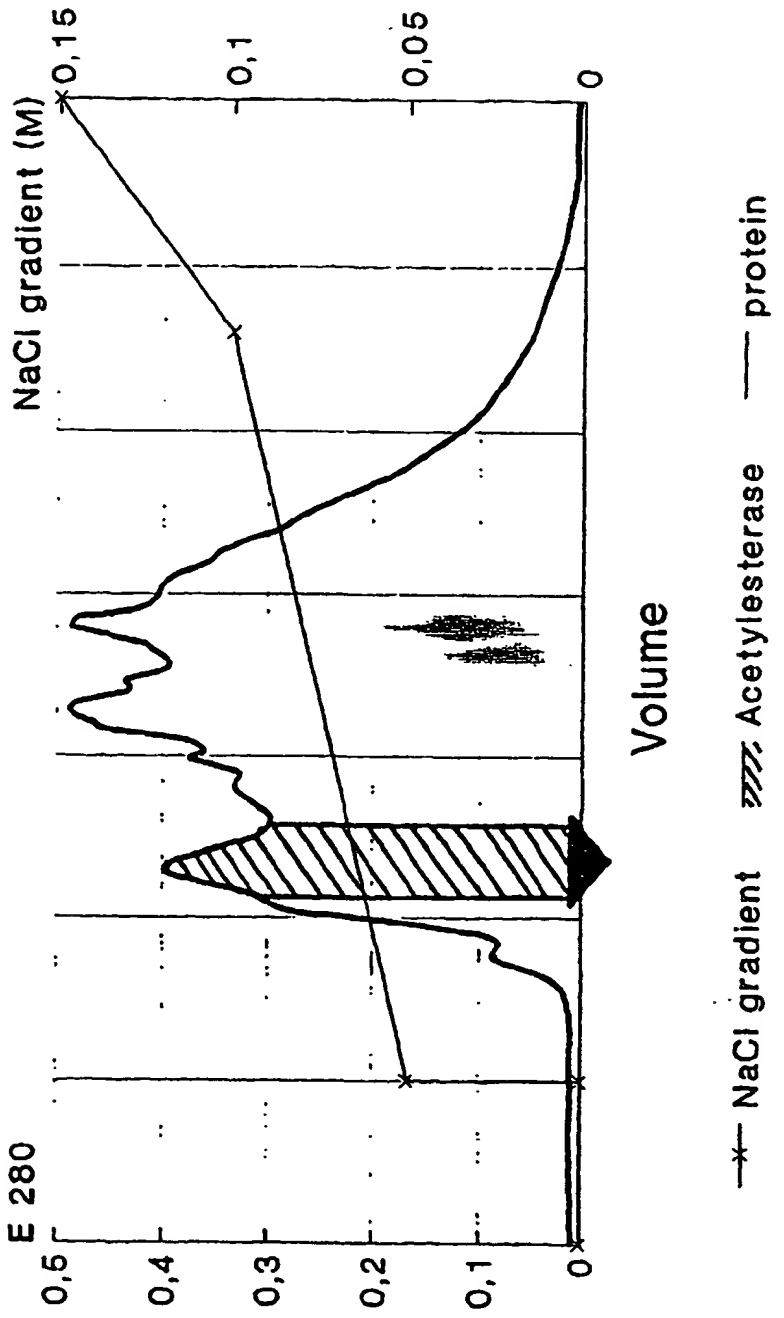
NaCl gradient (M)
0,15
0,1
0,05
0
E 280
0,5
0,4
0,3
0,2
0,1
0
Volume
NaCl gradient
Acetylesterase
protein

FIG. 4

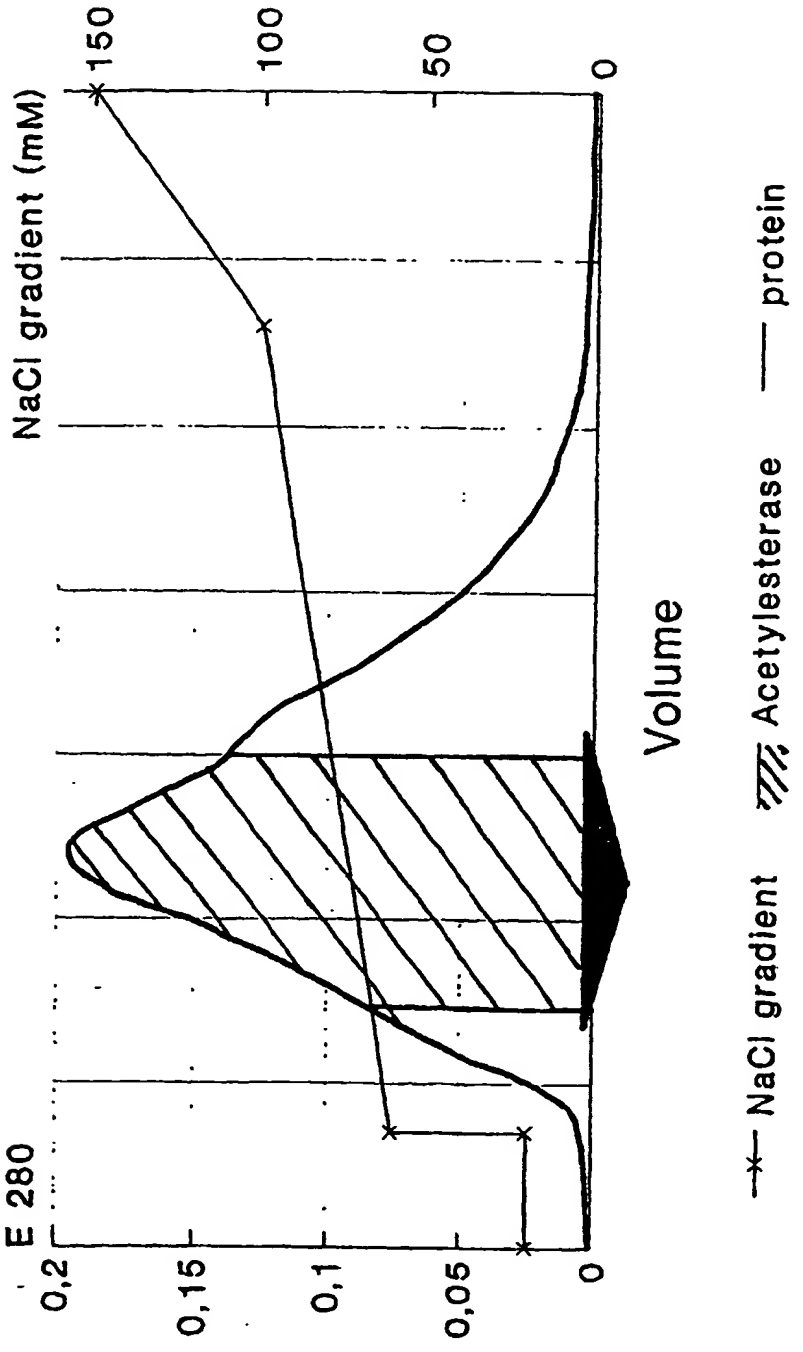
NaCl gradient (mM)
150
100
50
0
E 280
0,2
0,15
0,1
0,05
0
Volume
NaCl gradient
Acetylesterase
protein

FIG. 5

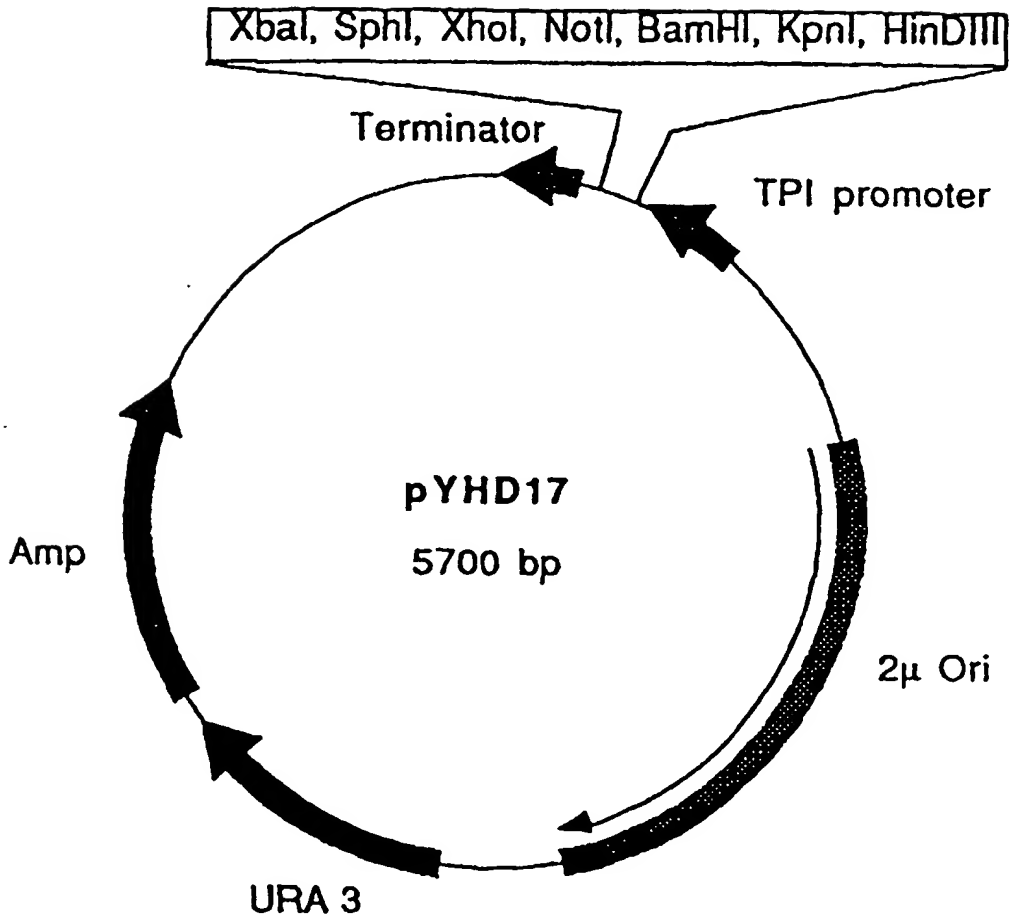
XbaI, SphI, XhoI, NotI, BamHI, KpnI, HinDIII
Terminator
TPI promoter
pYHD17
5700 bp
Amp
2μ Ori
URA 3

FIG. 6

```
                Asp Ser Thr Met Ala Lys Asn Gly Gly
5' - GGAATTCC GAC AGI ACI ATG GCI AAA AAC GGI GG -
3'               T TC             G   T
```

FIG. 7

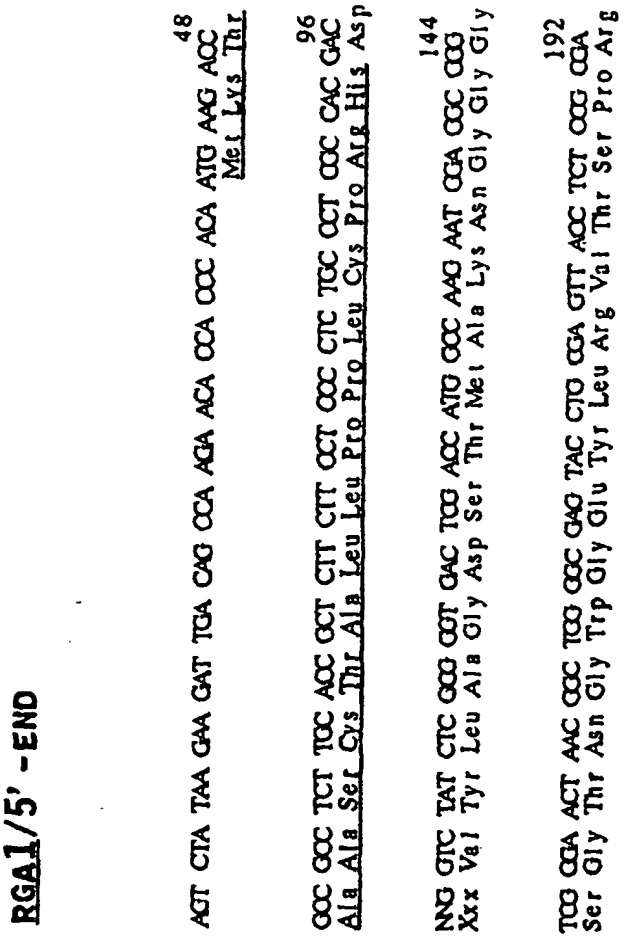
RGA1/5'-END
AGT CTA TAA GAA GAT TGA CAG CCA AGA ACA CCA CCC ACA ATG AAG ACC
48
Met Lys Thr
GCC GCC TCT TGC ACC GCT CTT CTT CCT CCC CTC TGC CCT CCC CAC GAC
96
Ala Ala Ser Cys Thr Ala Leu Leu Pro Pro Leu Cys Pro Arg His Asp
NNG GTC TAT CTC GCG CGT GAC TCG ACC ATG CCC AAG AAT CCA CCC CCG
144
Xxx Val Tyr Leu Ala Gly Asp Ser Thr Met Ala Lys Asn Gly Gly Gly
TCG CCA ACT AAC CCC TCG CCC CAG TAC CTG CGA GTT ACC TCT CCG CCA
192
Ser Gly Thr Asn Gly Trp Gly Glu Tyr Leu Arg Val Thr Ser Pro Arg
CAG TGG TTA ACG ACG CCG TCG CG
215
Gln Trp Leu Thr Thr Arg Ser Arg

FIG. 8

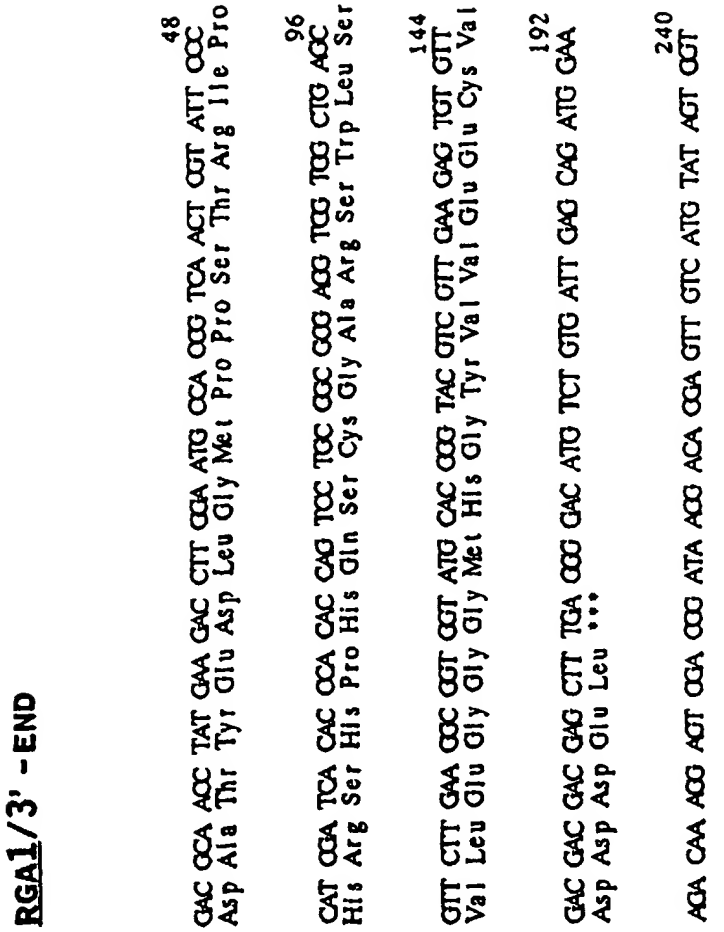
RGA1/3' - END
GAC GCA ACC TAT GAA GAC CTT CCA ATG CCA CCG TCA ACT CGT ATT CCC
Asp Ala Thr Tyr Glu Asp Leu Gly Met Pro Pro Ser Thr Arg Ile Pro
48
CAT CCA TCA CAC CCA CAC CAG TCC TGC CGC GCG ACG TCG TCG CTG AGC
His Arg Ser His Pro His Gln Ser Cys Gly Ala Arg Ser Trp Leu Ser
96
GTT CTT GAA CCC CGT CGT ATG CAC CCG TAC GTC GTT GAA GAG TGT GTT
Val Leu Glu Gly Gly Gly Met His Gly Tyr Val Val Glu Glu Cys Val
144
GAC GAC GAC GAG CTT TGA CCG GAC ATG TCT GTG ATT GAG CAG ATG GAA
Asp Asp Asp Glu Leu ***
192
AGA CAA ACG AGT CCA CCG ATA ACG ACA CGA GTT GTC ATG TAT AGT CGT
240
ATC GAC AAA AAA AAA AAA AAA AAA AAA AAA AAA AA
275

FIG. 9

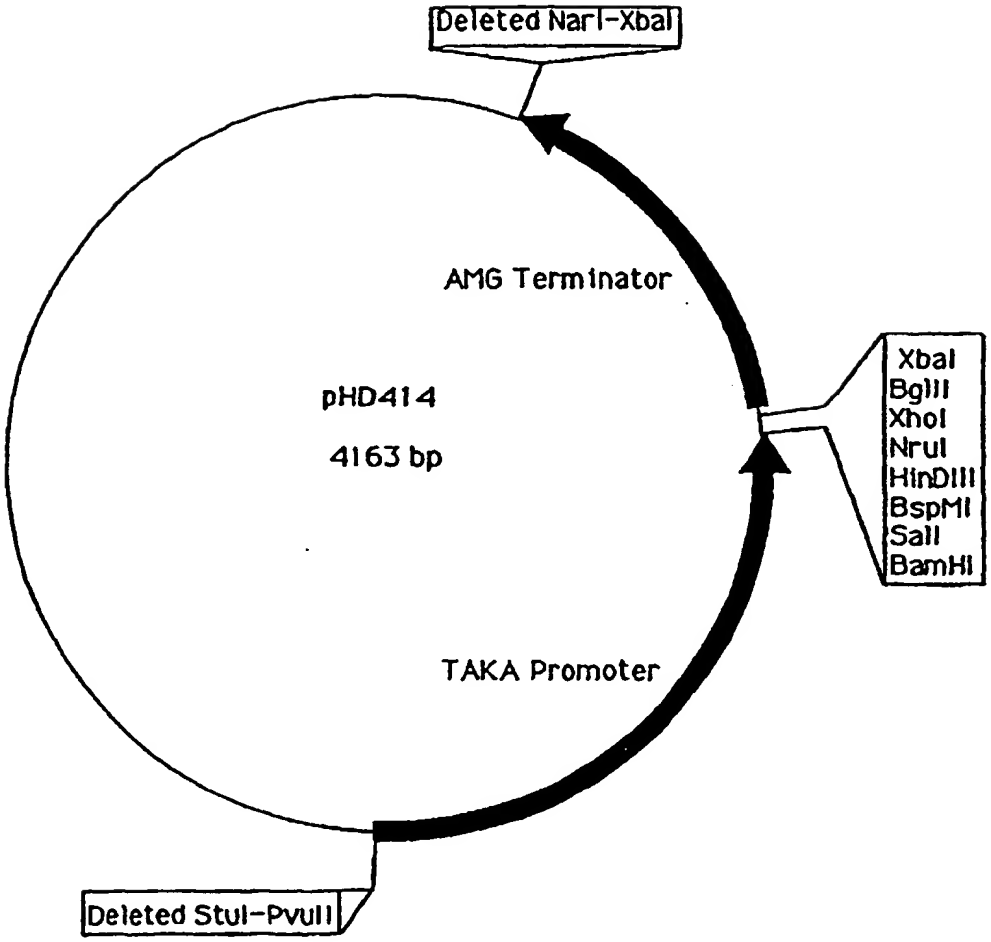
Deleted NarI-XbaI
AMG Terminator
pHD414
4163 bp
TAKA Promoter
Deleted StuI-PvuII
XbaI
BglII
XhoI
NruI
HinDIII
BspMI
SalI
BamHI

FIG. 10

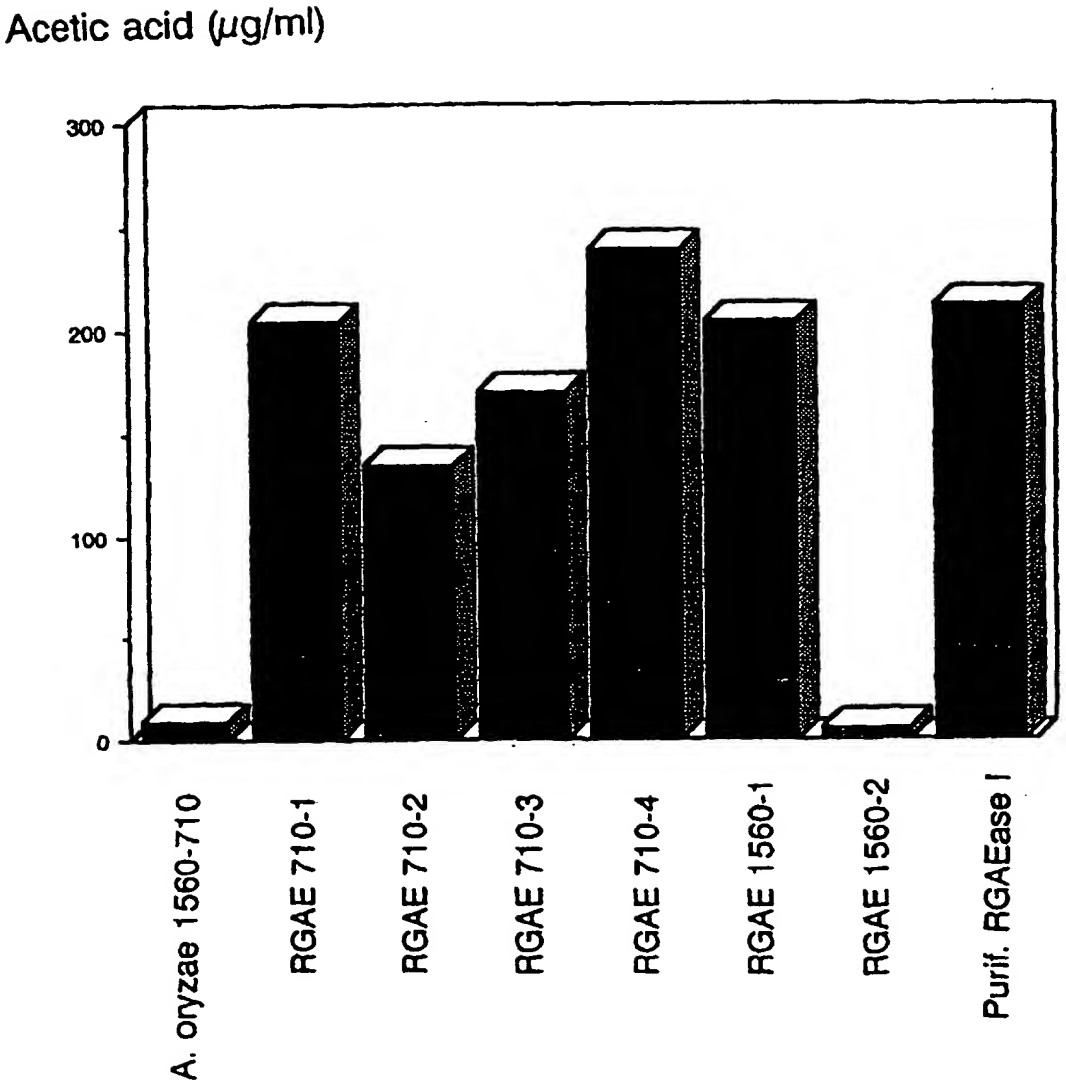
Acetic acid (μg/ml)
300
200
100
0
A. oryzae 1560-710
RGAE 710-1
RGAE 710-2
RGAE 710-3
RGAE 710-4
RGAE 1560-1
RGAE 1560-2
Purif. RGAEase I

FIG. 11